Nuclear Keratin 17 and its role in the DNA Damage Response

by

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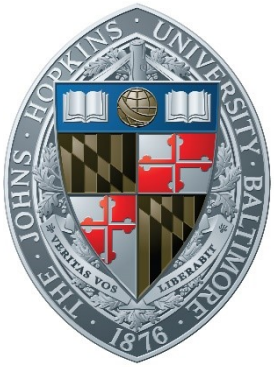

Abstract

Keratin 17 (*Krt17*; K17) is an intermediate filament protein known to be robustly upregulated in many types of epithelial-derived tumors. Recently, we have reported that K17 is capable of localizing to the nucleus of tumor-prone skin keratinocytes, where it can associate with chromatin, positively regulate the expression of pro-inflammatory chemokines and cytokines, and promote tumorigenesis. Given that K17 is known to be robustly upregulated in many types of epithelial-derived tumors and is required for timely tumor onset in two distinct transgenic mouse models for spontaneous skin tumorigenesis, we hypothesize that nuclear-localized K17 may play a role in regulating DNA damage and genome instability, a key enabler of early tumorigenesis. To investigate this possibility, we subjected human skin tumor cells lines, both parental and CRISPR/Cas9-generated *Krt17* null, to three molecularly distinct paradigms of DNA damage (1 ug of DMBA for 12 hrs, 100 uM H_2O_2 for 30 min, and 2 Gy ionizing radiation). In parental cells (expressing K17), all treatments triggered a robust DNA damage and repair response (DDR) as assessed by immunoblotting and immunostaining for DDR markers (including γH2AX pp53, pATM, pATR, pBRCA1, pCHK1, pCHK2), as well as by the comet assay to measure the extent of physical DNA damage. In striking contrast to parental skin tumor keratinocytes, we observed a significant attenuation of DDR for every readout in *Krt17* nulls across all three distinct DNA damage treatments. Re-expression of wildtype K17 into *Krt17* null cells completely restored the ability for DNA damaging agents to initiate the DDR. However, re-expression with wildtype K14, K16 and ΔNLS K17, which is a genetic variant lacking a functional nuclear localization signal, did not restore the ability of DNA damaging agents to induce the DDR. In addition, preliminary analysis suggests a chromatin organizational role for K17,

since K17 parental cells were more susceptible to micrococcal nuclease digestion, as opposed to the *Krt17* nulls. Taken together, these findings display a novel and specific role for nuclear K17 to regulate the DNA damage response in skin tumor keratinocytes, suggesting that K17 may act as a key regulator of both inflammation and genome instability during early tumorigenesis.

Thesis Committee: Dr. Pierre Coulombe and Dr. Fengyi Wan

Preface

This thesis is based on research conducted at the Johns Hopkins Bloomberg School of Public Health from June 2016 until June 2017. This year long project was conducted in the laboratory of Dr. Pierre A. Coulombe, where I worked under the supervision of both Dr. Coulombe and Dr. Ryan P. Hobbs, a senior postdoctoral fellow in the laboratory. All relevant literature was accessed through the Welch Library of the Bloomberg School of Public Health portal.

I would like to express a very sincere and grateful gratitude to Dr. Pierre Coulombe for his mentorship and guidance, and for allowing me to work on this project. It has been an absolute pleasure to be part of such an invigorating laboratory at such an exciting time.

To Dr. Ryan Hobbs, your mentorship and knowledge over the past year has taught me so much. I have acquired so much scientific research skills during my ScM program here at Johns Hopkins. I cannot thank you enough for the opportunity to work with you and learn from you.

To Justin Jacob, I thank you for all the times we have brainstormed on our research projects. You gave me an opportunity to work alongside with you as if I was a doctorate candidate myself.

To Dr. Beau Su, you have been a tremendous help to me as well as others in the laboratory, always there to troubleshoot problems with me.

I would also like to acknowledge Abigail Zieman and Fenrong Wang, for their insightful critique and wisdom on the experiments I have carried out over the past year.

To the rest of the Coulombe laboratory, I have enjoyed working with you over the past year and want to thank you for making the lab such an exciting place to be. I wish everyone the best in their future endeavors. Lastly, I would like to thank all the members of the department of Biochemistry and Molecular Biology of the Bloomberg School of Public Health for the help and support I have received over the past two years.

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LIST OF ABBREVIATIONS

A431	Human epidermoid carcinoma cell line
ATM	Ataxia telangiectasia mutated (gene:protein)
ATR	Ataxia telangiectasia and Rad3-related (gene:protein)
BCC	Basal cell carcinoma
BRCA1	Breast cancer susceptibility gene 1 protein
CHK1	Checkpoint kinase 1 (gene:protein)
CHK2	Checkpoint kinase 2 (gene:protein)
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMBA	7,12-Dimethylbenz(a)anthracene
DSB	Double-strand break
E2F	E2 transcription factor
EBS	Epidermolysis bullosa simplex
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gli2	GLI family zinc finger 2

hnRNP K	heterogeneous nuclear ribonucleoprotein K
HPV	Human papillomavirus
IF	Intermediate filament
IR	Ionizing radiation
K	Keratin protein
IFN-γ	Interferon gamma
kDa	Kilodalton
Krt	Keratin gene
MW	Molecular weight
nm	Nanometer
OCT	Optimal cutting temperature compound
P50, P120 etc.	Postnatal day 50, 120 etc.
P53	Tumor protein p53
PC	Pachyonychia congenita
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
Th1	Type 1 T helper cell
Th17	Type 17 T helper cell
Th2	Type 2 T helper cell
TNF-α	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WT	Wild-type
γH2AX	Phosphorylated histone variant H2AX
µg	Microgram
µL	Microliter

Introduction

Cancer: A Chronic Disease through the Ages

Cancer is not a simple disease. It is a chronic condition driven by genomic instability, which leads to an accumulation of DNA mutations, resulting in uncontrolled growth and proliferation of abnormal cells (Fearon & Vogestein, 1990). Genome wide analyses have demonstrated that this disease involves a plethora of atypical complex signaling pathways to ultimately abolish homeostatic conditions of the cell (Quigley et al., 2009). These conditions become fatal for the individual when the metastasis cannot be controlled and it leads to physiological stress and organ failure. Due to the complex nature of cancer, the medical and scientific community have been relentlessly studying the mechanisms of tumorigenesis.

Many efforts to combat the disease are through preventative measures, such as mammography screening for breast cancer and colonoscopy screening for colon cancer. These measures will allow earlier diagnosis of the cancer and can be immediately treated at its early phases. By detecting a cancer in its early stages, there is a much higher success rate for removing the tumor with no recurrences. Cancer staging is based on the size of the tumor or if it has spread to adjacent lymph nodes. Generally, if the cancer cells still reside in the layer of cells where they developed and haven't spread, they are staged as *in situ.* However, if the cancer cells have penetrated beyond the original tissue, the cancer cell is deemed invasive. Clinically, the TNM staging system is used (Edge et al., 2010). It assesses cancer growth and spread by evaluating the extent of the primary tumor (T), absence or presence of regional lymph node involvement (N), and absence or presence of distance metastases (M). Once a T,

N, or M category is assigned, the stage is then assigned. It can be stage 0, which is *in situ,* stage I being early, and stage IV being the most advanced disease stage.

These different stages of cancer metastasis are dependent on the aggressiveness of the tumor, which is reliant on the six hallmarks of cancer: 1) evading apoptosis; 2) self-sufficiency in growth signals; 3) insensitivity to anti-growth signals; 4) tissue invasion & metastasis; 5) limitless replicative potential; 6) sustained angiogenesis. (Hanahan & Weinberg, 2000). Each of these hallmarks are representative of characteristics that ignore cellular homeostatic conditions to induce aggressive progression of a tumor. Recent data suggests that there are additional attributes that are functionally important for cancer development (Hanahan & Weinberg, 2011). These new attributes are separated into two categories: Emerging Hallmarks and Enabling Characteristics. The first emerging hallmark of cancer is the capability for cancer cells to alter its metabolic pathway to ensure neoplastic proliferation, and the second is the ability to evade immune surveillance from lymphocytes. In addition, cancer cells contain high levels of genomic instability, which results in DNA mutations, giving these cells to drive tumor progression. This enabling characteristic is accompanied by cytokine expression by the innate immune system, which may trigger tumor-promoting inflammation.

DNA damage and its role in carcinogenesis

As such, genetic instability and inflammation are key enablers of tumor initiation. Genetic instability is attributed to DNA damage, caused by various agents that are capable of disrupting the structure of nucleic acids (O'Connor, 2015). Such examples of DNA damaging agents include chemical carcinogens, oxidative reagents, and external sources of ionizing radiation. DMBA is a polycyclic aromatic hydrocarbon, a potent carcinogen that is known to have mutagenic properties on DNA after exposure, forming various DMBA-DNA adducts

(Dipple et al., 1984; Slaga et al., 1974; Ward et al., 1986). More importantly, DMBA is a commonly utilized as initiating agents in the two-step carcinogenesis experimental protocol (Abel et al., 2009). Another agent that is known to initiate DNA damage is hydrogen peroxide, where it is commonly known to create free radicals known as reactive oxygen species (ROS) when metabolized by the cells. These ROS are known to cause significant damage to the DNA as well, by abstracting the electrons on nitrogenous bases, resulting strand breaks and alterations in the DNA (Burdon et al., 1996). Likewise, ionizing radiation (IR) can cause DNA damage as well, by directly inducing both single-strand breaks (SSB) and double-strand breaks (DSB) in the DNA double helix backbone. If misrepaired - for example, the inaccurate rejoining of broken DNA ends at DSBs - such breaks can induce mutations and lead to widespread structural rearrangement of the genome (Lord & Ashworth, 2012).

Evoking DNA damage will trigger the DNA damage response (DDR), which is a collective term for the plethora of different intra- and inter-cellular signaling events and enzyme activities that result from the induction and detection of DNA damage. These include events that lead to cell-cycle arrest, regulation of DNA replication, and the repair or bypass of DNA damage. Should DNA repair not be possible or subpar repair leads to a continuous level of genomic instability, DDR can also impact on cell fate decisions, such as cell death or senescence (d'Adda di Fagagna et al, 2003; Kang et al., 2015).

Recent analysis suggests that there are over 400 proteins essential to DDR (Pearl et al., 2015), each playing a significant role in the signaling cascade of DDR. A canonical DDR pathway has been established, where, upon insult, the MRE11-RAD50-NBS1 complex will detect damage and phosphorylate two central transducers, ATM and ATR (Zou and Elledge, 2003). When phosphorylated, these two proteins will act as kinases and phosphorylate H2AX,

BRCA1, CHK1, and CHK2 (Figure 1). These activated kinases will further phosphorylate targets such as p53 and CDC25 to influence the fate of the cell.

The phosphorylation of histone variant H2AX on serine-139, also referred to as γH2AX, rapidly occurs upon DNA damage, in both single-strand and double-strand breaks (Sharma et al., 2012). The phosphorylation event of this histone variant creates foci within the cell nucleus, indicative of the number of DNA breaks, as well as the extent of the damage (Lobrich et al., 2010) The purpose of γH2AX is to modify chromatin structure and associate with other DDR proteins, which also includes the repair machinery, allowing it access to initiate DNA repair. γH2AX as a marker of DNA damage has been thoroughly reported and utilized in a variety of experimental assays such as immunostaining, immunoblotting, all which have reported the efficiency and reliability of this moiety as a marker for DNA damage (Mah et al., 2010; Sharma et al., 2012). Thus, the levels of γH2AX under these experimental conditions is an accurate indicator of DNA damage.

On the topic of DDR, there has also been further evidence showing a crosstalk between chromatin state and the DDR (Sulli & Di Micco & d'Adda di Fagagna, 2012). Chromatin can affect the sensitivity of DNA to DNA-damaging agents. Evidence for this has come from studies showing that, on exposure to ionizing radiation, DNA that is depleted of histones, or of other chromatin-associated proteins, promoting a euchromatin state resulted in more lesions compared with compacted chromatin (Costes et al., 2007; Elia & Bradley, 1992). It is clear that in addition to physically shielding DNA from damage, the chromatin structure also affects local DDR signaling around DNA damage. For example, heterochromatin seems to be resistant to γH2AX induction, whereas a shift to a euchromatin state promotes γH2AX induction (Di Micco et al., 2011; Kim et al., 2007). Furthermore, decreasing the degree of heterochromatin

structure by inhibiting histone deacetylases (HDACs), which decreases chromatin compaction by removing acetyl groups from histones, or by reducing the levels of histone H1, enhances DDR signaling and the extent of γH2AX activation (Cowell et al., 2007; Murga et al., 2007).

Other recent studies such as the one carried out by Wu et al. (2010) reported that the loss of two E3 ubiquitin ligases, RNF8 and Chfr, are vital to the DDR by controlling H4 Lys 16 acetylation though MRG-dependent acetyltransferase complexes, promoting chromatin relaxation, hence allowing recruitment and activation of ATM and other DNA damage response moieties. To further substantiate this complex interplay between chromatin architecture and the DNA damage response, a recent group from Cambridge has reported that the DNA damage response is modified through two histone deacetylases: HDAC1 and HDAC2, specifically altering the state of H3 Lys 56 acetylation and H4 Lys 16 acetylation (Miller et al., 2010). In addition, the data showed that cells depleted of histone deacetylases were hypersensitive to DNA-damaging agents and showed sustained DNA damage signaling, as reflected upon a lack of non-homologous end-joining (NHEJ) repair mechanism. This suggests that HDACS, whose canonical role in physiology is chromatin organization, are able to modulate the DNA damage response by promoting Double strand breaks (DSB) repair.

Overall, chromatin conformation can be hypothesized as an important component of the activation of DDR signaling, which then plays a role in determining the fate of the cell. Interestingly, a common characteristic of many tumor types is the euchromatic nature of their chromosomes, suggesting that cancer cells, through the expansion of the euchromatin structure, may allow heightened sensitivity to damaging agents, as well as oncogene activation (Sulli, Di Micco & di Fagagna, 2012).

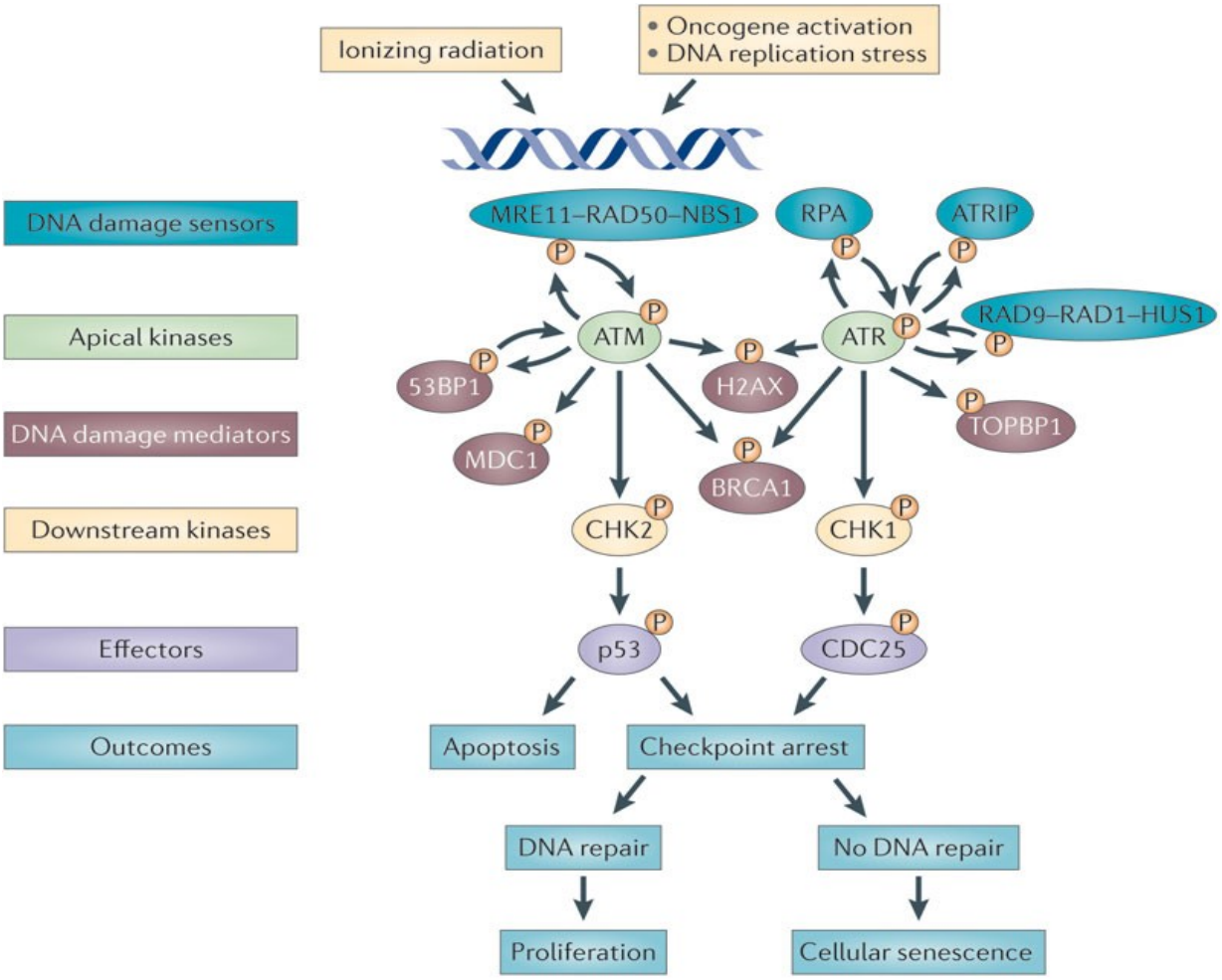

Figure 1. The DNA damage response. The DNA damage response (DDR) pathway is composed of two main DNA sensors: the MRN complex (MRE-11-RAD50-NBS1) and the RAD9 complex (RAD9-RAD1-HUS1), where they detect double-stranded breaks and single-stranded breaks, respectively. These sensors of damage then recruit the apical kinases ATM (through the MRN complex) and ATR (through RAD9 complex) for activation, which in turn, will phosphorylate histone variant H2AX on serine 139. ATM is seen activated more by DSB and ATR is more responsible for other types of genotoxic stress such as single-stranded breaks. γH2AX is required to recruit MDC1 to amplify DDR signaling via a positive feed-back loop between ATM and the MRN complex. Other DDR moieties are recruited and phosphorylated as well, such as CHK1 and CHK2, which in turn will activate downstream effectors such as p53 and CDC25 that modulates cell cycle arrest and apoptosis. Figure adapted from Sulli, Di Micco & di Fagagna, 2012.

Keratins: A novel family of intermediate filaments

Most eukaryotic cells contain in their cytoplasm an elaborate cytoskeletal system consisting of intermediate filaments (IFs), which are chemically very stable and long filaments of ~10 nm in diameter (Fuchs & Cleaveland, 1998; Kim & Coulombe, 2007). Among the various families and subfamilies of IF proteins, keratin genes represent the largest group with 54 distinct functional genes (28 type I and 28 type II) which are highly expressed in epithelial type cells and regulated in a cell-type and context-dependent manner (Schweizer et al., 2006; Sun et al, 1983). The classification of keratins as type I and type II IFs is based on basic features such as gene substructure and sequence homology (Figure 2A; Coulombe, Bernot & Lee, 2013; Fuchs and Marchuk, 1983; Moll and Divo, 2008).

Keratins readily self-assemble into 10 nm filaments *in vitro* and *in vivo* and localize to the cytoplasm under normal physiological conditions (Figure 2C; Gu & Coulombe, 2005; Coulombe and Omary, 2002; Yamada et al., 2002). Keratins have a tripartite structure with an N-terminal head, a central rod, and C-terminal tail domains (Figure 2B; Coulombe, Bernot & Lee, 2013; Bragulla & Hoberger, 2009). Keratin IF proteins have molecular weights ranging from 40 to 70 kDa (Schweizer et al., 2006; Sun et al., 1983). Keratin filaments are obligatory heteromeric assemblies with type I and II moieties occurring in a 1:1 molear ratio. The central rod domain of keratins features long-range heptad repeat of hydrophobic amino acids that allow the formation of coiled-coil interaction to allow pairing between type I and type II keratins (Coulombe & Fuchs, 1990; Hatzfeld &Weber, 1990). It is important to understand that the expression of type I and type II keratin genes is coordinately regulated in a differentiation and context-specific fashion specific in epithelial cells and tissues. For example, the type II *Krt5* and type I *Krt14* are expressed together in the basal progenitor layer of epidermis and

related complex epithelia, whereas type II *Krt8* and type I *Krt18* are expressed in simple epithelia such as the gut, liver, kidneys, and others (Coulombe & Fuchs; 1990).

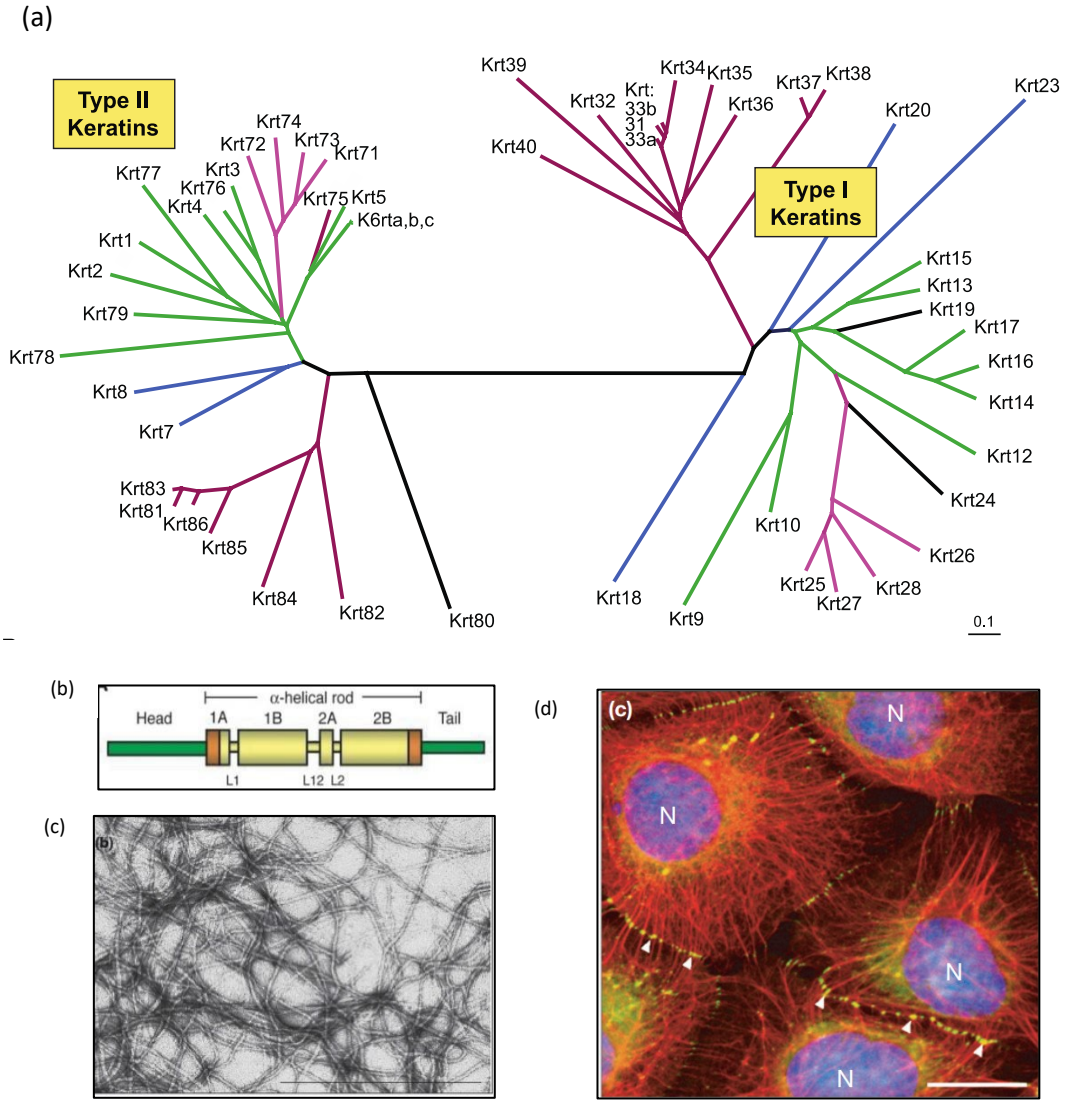

Figure 2. The keratin structure and its organization. (a) The keratin proteins are organized into type I and type II keratins based on primary protein sequence homology. (b) Schematic of keratin secondary structure. All keratins share a tripartite structure consisting of a head, tail, and central α-helical rod domain that plays in the role of self-assembly. (c) Electron microscopy image of *in vitro* reconstructed filament from purified keratin 5 and keratin 14 proteins. Scale bar = 500 µm. (d) Triple-labeling for keratin (in red) and desmoplakin, a desmosome component (in green) and DNA (in blue) by indirect immunofluorescence of epithelial cells in culture. Scale bar = 30 µm. Figure adapted from Gu & Coulombe, 2005; Coulombe, Bernot & Lee, 2013.

Functional roles of keratins

It has now long been established that keratin intermediate filaments are vital for the mechanical stability and integrity of epithelial cells and tissues (Fuchs and Coulombe, Cell 1992; Gu & Coulombe, 2007). Although type I and type II keratins are absolutely essential in maintaining cellular architecture, as was originally established in the setting of a rare disorder, epidermolysis bullosa simplex (EBS) (Fuchs and Coulombe, Cell 1992; Coulombe, J Invest Dermatol 2017). Disease causing mutations such as frameshift mutations and point mutations affecting the tail domain encoding exon of epidermal keratins are associated with different forms of epidermolysis bullosa simplex (EBS), epidermolytic hyperkeratosis (EHK) and other related diseases (Gu et al., 2003). EBS and EHK are classical skin fragility disorders that result from genetic defects in keratin proteins and the keratin network of specific subpopulations of keratinocytes in the epidermis. It was discovered most EBS are due to dominantly acting mutations in either K5 or K14, whereas K1 and K10 are most mutated in EHK (Fuchs and Coulombe, Cell 1992; Omary et al., NEJM 2004).

Additional, non-canonical functions have been identified for keratin proteins, such as their ability to interact with organelles through a number of mechanisms. Recent data has showed keratins to regulate the positioning and morphology of organelles such as the mitochondria, subsequently affecting their functionality (Toivola et al., 2005). Keratin proteins also have regulatory functions and are involved in intracellular signaling pathways, e.g. protection from stress, wound healing, inflammation, cell growth and apoptosis (Moll & Divo, 2008; Paladini & Coulombe; Chung et al, 2015). A specific keratin that has been implicated in a variety of cellular signaling processes is the type I protein, keratin 17 (K17), as detailed in the next section.

Physiological roles of keratin 17

Keratin 17 is a type I keratin of 46 kDa in mass (432 amino acids in the human; Troyanovsky et al., 1992). At baseline, the *KRT17* gene is constitutively expressed in ectoderm-derived epithelial appendages such as the hair follicle, nail beds, tooth, thymus, and various glandular epithelias. Furthermore, *KRT17* is induced upon woulding for tissue repair, which along with carcinomas is a setting of enhanced cell proliferation and altered differentiation (Mansbridge & Knapp, 1987; McGowan & Coulombe, 1998). The bulk of information available on K17 functions in normal physiology is derived from the research performed in a *Krt17* null mouse model by McGowan & Coulombe (2002). Structurally, K17 protein has been reported to play a role in the postnatal hair follicle, by providing mechanical support to the hair shaft and maintaining the viability of hair-producing epithelial cells (McGowan & Coulombe, 2002). In addition, K17 influences apoptotic signaling, with studies that reveal a relationship between K17 and TNF-α to regulate hair follicle cycling. TNF-α is a powerful and pleiotropic signal with a vast array of physiological functions such as eliciting the immunological response and promoting apoptosis. K17 has been reported to modulate TNF-α signaling in hair follicles likely through its interaction with an adaptor protein called TRADD, which promotes their persistence in the anagen, growth-promoting state (Tong & Coulombe, 2006).

Additional studies such as the one carried out by Kim et al. (2006) described a new function of K17, where it was reported to impact the signaling pathway and regulation of cell growth by binding directing with adaptor protein 14-3-3σ to stimulate the mTOR pathway, most notably regulating protein synthesis and epithelial cell growth post-wounding (Kim et al,, 2006). Imbalance of mTOR function has been associated with many classes of cancers such as

colon, gastric and hepatocellular carcinomas, and has a significant effect of tumor progression (Kim & Eng 2011; Populo et al., 2011; Villaneuva et al., 2008) So far, the functional relavance of K17 is not understood fully yet, but emerging evidence on several fronts converge in establishing that K17 is playing a significant role in various signalling events that impacts cell growth and proliferation.

Keratin 17 and its role in tumorigenesis

There has been a vast body of work that indicates K17 plays a functional role in tumorigenesis. Recent evidence showed that high expression of K17 is correlated with the expression of epidermal growth factor receptor (EGFR), which is known to be associated with poor prognosis through Kaplan Meier survival analysis in the head and neck, ovarian, cervical and esophageal cancers (Bauknecht et al., 1989; Ozawa et al., 1980; Wang et al., 2013). Furthermore, these investigators also noted that K17 expression correlates with the level of tumor aggression, as well as its progression. Preliminary research on this intermediate filament noted K17 to be prominent in basal cell carcinomas (BCC), observed by Markey et al. (1992) through a study of utilizing immunohistochemistry staining to visualize K17 expression *in situ* (also, see Moll et al., 1982). In addition, a clinical study performed by Goyal et al. (2016) also noted high expression of K17 in cutaneous lymphadenoma (CL), which is a rare neoplasm that is clinically and histologically similar to BCC. Overall, K17 expression appears to be prominent in many disease settings, such as cervical carcinomas, where the level of K17 expression is both a reliable diagnostic and prognosis biomarker.

Investigation of K17 in other pathologies has provided more detailed insight into the molecular mechanisms by which K17 contributes to cancer. Early studies by Liao et al. (2016) utilized a murine model of radiation dermatitis, and sought to understand the role K17 plays in

skin epithelia under the exposure of ionizing radation. Results indicated that K17 expression is initially down-regulated after initial ionizing radiation treatment and is up-regulated upon after chronic exposure to ionizing radiation. The down-regulation correlated with the activation of p53 signaling, hence the investigators predicted a relationship between K17 and p53 (Liao et al., 2016). Deletion of p53 abolishes the initial down-regulation of K17 upon damage, but not its up-regulation, which may suggest p53 suppresses K17 transcription, as opposed to suppression through post-translational modification. Taking advantage of chromatin immunoprecipitation (Co-IP) and electromobility shift assay, results identified two p53 binding sites in the promoter region of *KRT17*, leading to the concludion that in response to ionizing radiation p53 works to suppress *KRT17* expresson.

Recent evidence by DePianto et al. (2010) showed that BCC development is due to K17 expression. A transgenic model in mice for BCC was developed whereby the transcription factor Gli2 is constitutively expressed using the KRT5 gene promoter, leading to sustained activiation of the Sonic hedgehog (Shh) signaling pathway and development of basaloid skin tumors (Grachtchouk et al., 2000). Interestingly enough, the genetic ablation of *Krt17* in the setting of this $Gli2^{tg}$ mouse model significantly attenuates the extent of basal cell-like lesions (Figure 3a; DePianto et al., 2010). Specifically, loss of *Krt17* markedly delays the onset of tumor in ear skin of $Gli2^{tg}$ mice (Figure 3b; DePiantoet al., 2010). Loss of *Krt17* in this setting also results in reduced tumor keratinocyte proliferation and inflammatory cytokine expression. qRT-PCR analysis indicated a shift of cytokine expression from pro-inflammatory to anti-inflammatory, further complementing these findings. In addition, this phenomenon was K17 specific, since genetic ablation of *Krt14* did not display a significant impact in the the onset of tumorigenesis (Figure 3c; DePianto et al., 2010).

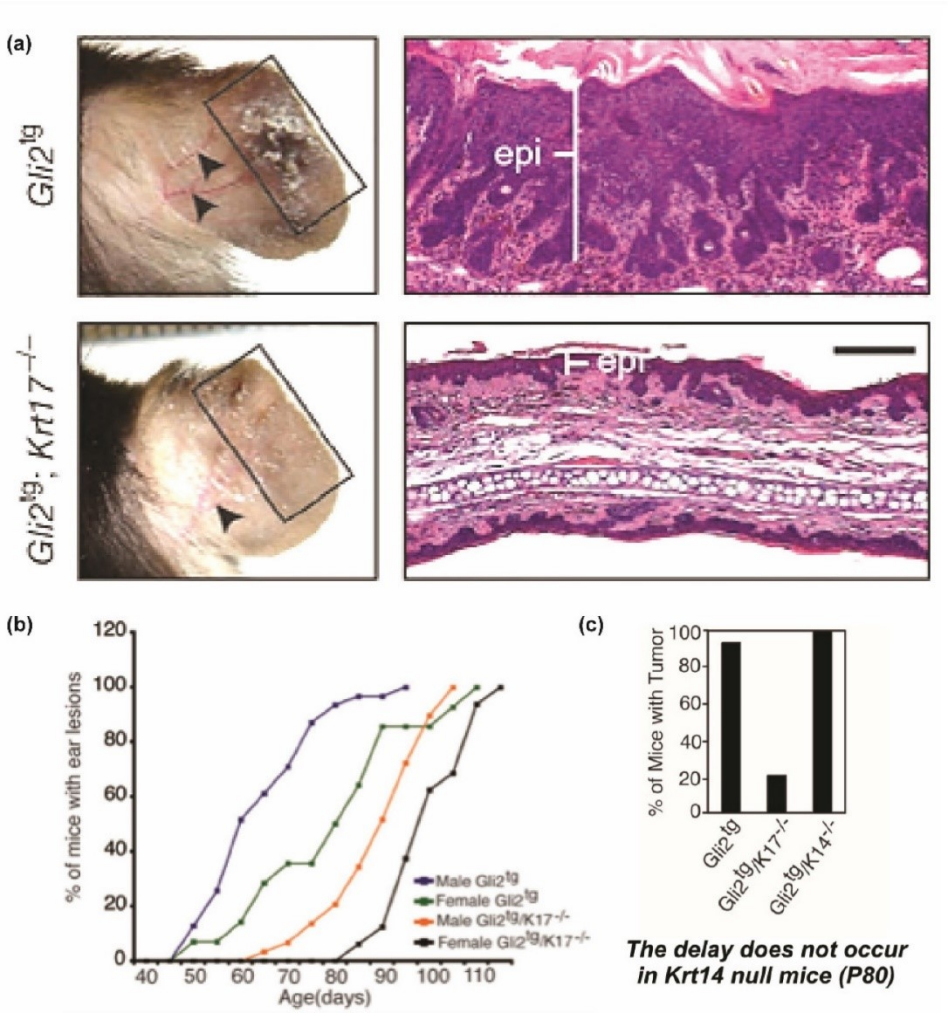

Figure 3. Genetic ablation of K17 delays tumor onset of basal cell carcinoma in ear epithelia. (a) Left panel indicates that P80 $Gli2^{Tg}$ mice spontaneously develop ear lesions, but the onset of BCC-like lesions is delayed in the K17 null mice. Right panel is a hematoxylin-eosin stained ear tissue showing a dramatic reduction of thickness of the ear upon genetic ablation of K17. (b) Percentage of mice with ear lesions divided among sexes and genotype within a P40-P120 timeframe displayed a delayed tumor onset upon genetic ablation of K17 but no difference in the delay among the sexes. (c) Quantification of percentage of mice with tumor seen in K17 null at P80 and representative of this as a K17 specific event when compared with the K14 null mice. Figure adapted from DePianto et al., 2010.

Building upon this work, a separate transgenic mouse model for squamous cell carcinoma (SCC) was developed by Arbeit et al. (1994) and also exploited by the Coulombe laboratory to further investigate K17's role in tumorigenesis. This model entails the expression of the open early genes of human papillomavirus type 16 (HPV16) using the *KRT14* gene promoter and elicits the formation of SCC-like lesions in ear skin (Arbeit et al., 1994; Munoz et

al., 2006). Of note, this model is is different genetic strain background (FVB) relative to the Gli2^{tg} model (C57Bl/6). Hobbs et al. (2015) recently reported that genetic ablation of *Krt17* in the HPV16$^{Tg/+}$ setting (HPV16$^{Tg/+}$ *Krt17*$^{-/-}$) dramatically reduced epidermal thickness in the ear tissue, complemented with a reduction of pro-inflamatory cytokine induction and mitotic activity (Figure 4; Hobbs et al., 2015). Follow-up efforts to investigate the molecular mechanisms whereby K17 impacts tumorigenesis and inflammation showed a physical and functional interaction between K17 and AIRE (autoimmune regulator), a transcriptional regulator, and between K17 and hnRNPK, a ribonucleoprotein, which both influence the level of cytokine gene expression (Hobbs et al, 2015; Chung et al., 2015).

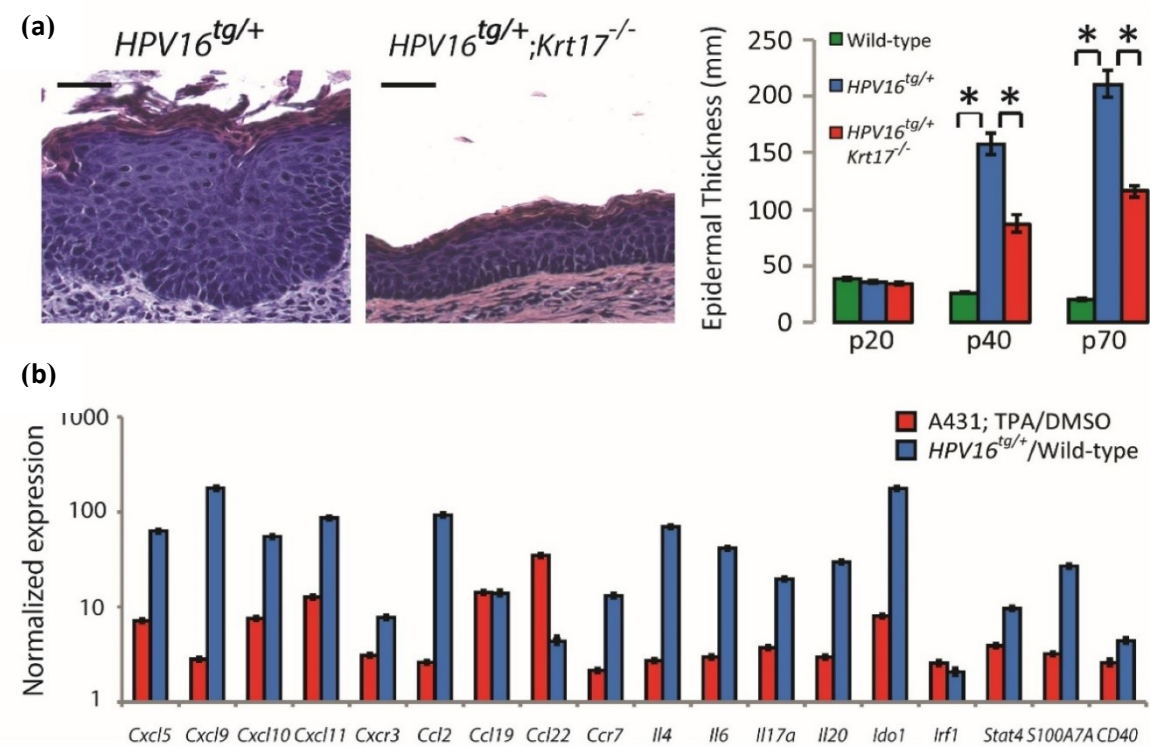

Figure 4. Loss of K17 attenuates squamous cell carcinoma development. (a) Hematoxylin-eosin stain of HPV16$^{Tg/+}$ and HPV16$^{Tg/+}$; *Krt17*$^{-/-}$ mouse ear sections display a significant increase in epidermal thickness. Quantification of epidermal thickness at both pre-lesional and lesional time points between wild-type, HPV16$^{Tg/+}$, and HPV16$^{Tg/+}$; *Krt17*$^{-/-}$ are seen on the right. All scale bars = 20 μm. (b) TPA treated tumor keratinocytes (A431) in culture showed an induction of pro-inflammatory cytokine expression relative to DMSO treated keratinocytes. HPV16$^{Tg/+}$ ear skin tissue showed higher levels of pro-inflammatory cutokine expression as well relative to WT ear skin tissue. Figure adapted from Hobbs et al., 2015.

Keratin 17 localizes into the cell nucleus

While investigating the mechanisms underlying the ability of K17 to regulate the expression of pro-inflammatory cytokines, Hobbs et al. (2015) also observed that K17 can translocate into the nucleus of human and mouse tumor keratinocytes . A similar observation was made by Escobar-Hoyos et al. (2015), whereby K17, functioning as an oncoprotein in the cervix, breast, and pancreatic cancer cells, is able to regulate the subcellular localization and degradation of p27KIP1, a protein that inhibits the activity of cyclin-dependent kinases, thus halting the cell cycle. K17 is able to translocate into the nuclus via a nuclear localization signal (NLS) to bind p27KIP1 for degradation. Depletion in endogenous p27KIP1 prompts the cellular machinary to continue dividing, resulting in tumorigenesis. In both of these studies, uncovering the presence of nuclear K17 in cultured tumor kerationcyes involved a potent inhibitor of CRM1/Exportin 1 mediated nuclear transport, Leptomycin B (LMB). This chemical compound is able to trap nuclear K17 inside the nucleus and facilitate the detection through fluorescent microscopy. Others have also adapted this strategy in visualizing cytoskeletal associated proteins, such as lipoma preferred partner, zyxin, ZO-2, and KEAP1 (Petit et al., 2000; Nix et al., 2001; Islas et al., 2002; Velichkova & Hasson, 2005).

Importantly, the NLS is a di-lysine motif located at lysines 399 and 400 in the tail domain moiety of human K17, which is highly conserved between humans and primates, rodents, amphibians and fish (Hobbs & Jacob, 2015). Cultured cell lines transiently transfected with K17 engineered by site-directed mutagenesis to contain an alanine instead of a lysine at residue 400 (ΔNLS K17) exhibited attenuated localization into the nucleus and a reduced ability to upregulate the expression of K17-dependent pro-inflammator cytokine (Hobbs et al., 2015).

The limited knowledge accumulated to date regarding K17's presence in the nucleus suggests that it may play multiple roles within the nucleus, as it has been seen to do so previously in the cytoplasm to regulate the homeostatic functions of the cell (Kim et al., 2006); McGowan et al., 2002; Tong & Coulombe, 2006). As reported by Hobbs et al. (2015), nuclear K17 associates with the promoter region of cytokine genes, as well as AIRE and the p65 subunit of NF-kappaB. Taken together, the data suggest potential nuclear roles carried out by K17, such as binding to chromatin and transcription factors to regulate gene expression.

RESULTS

Previous finding: Loss of K17 does not attenuate the DDR in the HPV mouse model

As previously shown, the HPV16$^{Tg/+}$ model is a well established paradigm for the development of epithelial lesions and subsequent squamous cell carcinomas that coincides with the pathophysiology observed in human SCC (Arbeit et al., 1994; Hobbs et al., 2015). This model was further utilized to investigate whether K17 expression correlated with and is required for an increased DNA damage and response. Previous research indicated that lesion development occurs between P60 and P120 in the HPV16$^{Tg/+}$ ear tissue, with complete penetrance by P120 (Hobbs et al., 2015). From this piece of data, P50 was chosen as a pre-lesional time point to assess levels of DNA damage and its associated response. Although both K17 and DDR associated proteins were both found to be robustly upregulated in the ear, tail, and back skin tissue of *HPV16$^{Tg/+}$* mice relative to that of *WT* control mice, the DDR was not observed to be K17-dependent at this time point *in vivo*. (unpublished data from Stefan Prendergast, 2016). From this, we speculated that K17-dependence could occur at distinct time points (earlier?) or in a distinct paradigm for initiating and promoting DNA damage . Thus, we decided to utilize to apply acute DNA damage protocols with an initial focus on *in vitro* cell culture experiments, using three different modalities to trigger a DNA damage response: Ionizing radiation (IR) for inducing double-strand breaks, 7,12-Dimethylbenz(a)anthracene (DMBA) for creating DNA-adducts, and hydrogen peroxide (H_2O_2), which is known to create both single-strand and double-strand breaks.

Stimulation of K17 expression upon treatment with both DMBA and H_2O_2

As a means of determining whether K17 is induced upon acute DNA damage, mouse keratinocytes in primary culture derived from male mice (P2, *Krt17$^{+/+}$)* were isolated, treated

with either 1 ug of DMBA for 12 hours, 100 μM of H_2O_2 for 30 minutes, or the mock treatment (acetone). Indirect immunofluorescence microscopy images stained for K17 suggests that K17 expression is induced upon treatment with DMBA and H_2O_2 compared to acetone control (Figure 5). Fluorescence signal quantitation shows that K17 protein levels are increased in treated keratinocytes compared to the control ($p<0.005$; Mock vs DMBA, $p=0.002$; Mock vs H_2O_2, $p=0.003$). This finding, along with the findings of unpublished data from the Coulombe lab, suggests that K17 may play a role in the initiating events of acute DNA damage. Further substantiating this finding, a similar experiment was carried out *in vivo* as well. Ear tissue sections from two-month-old female mice (*Krt17*$^{+/-}$or *Krt17* $^{-/-}$) that had been treated with either 20 μg of DMBA or acetone (control) on the dorsal surface were immunostained for K17. The results (two biological replicates) revealed a significant induction of K17 when DMBA is applied (Figure 6).

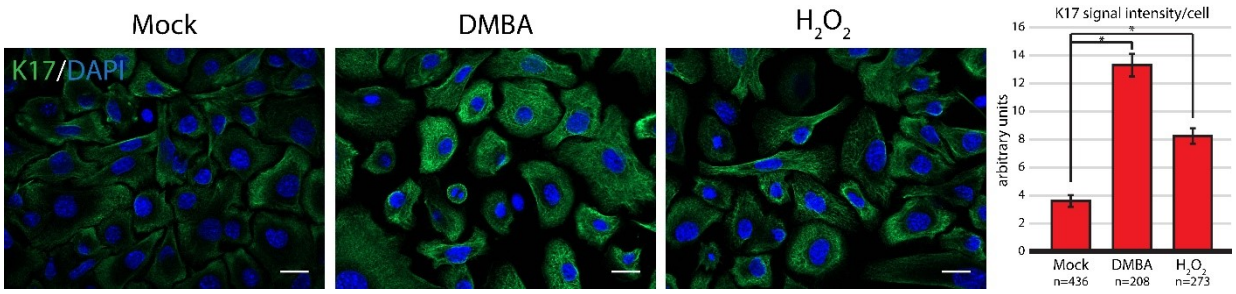

Figure 5. K17 induction in mouse keratinocytes upon DNA damage agent insult. Mouse keratinocytes in primary culture (from 2 day old neonates) were seeded on glass cover slips, treated with 1 μg of DMBA (12 hr) or 100 μM H_2O_2 (30 min), fixed with 4% paraformaldehyde, permeabilized with 0.2% triton, and immunostained for K17 (indirect immunofluorescence). All scale bars = 20 μm. Signal intensity per cell is quantified in the graph to the right using ImageJ and Excel. N= number of cells. *$p<0.005$ (Student's t-test).

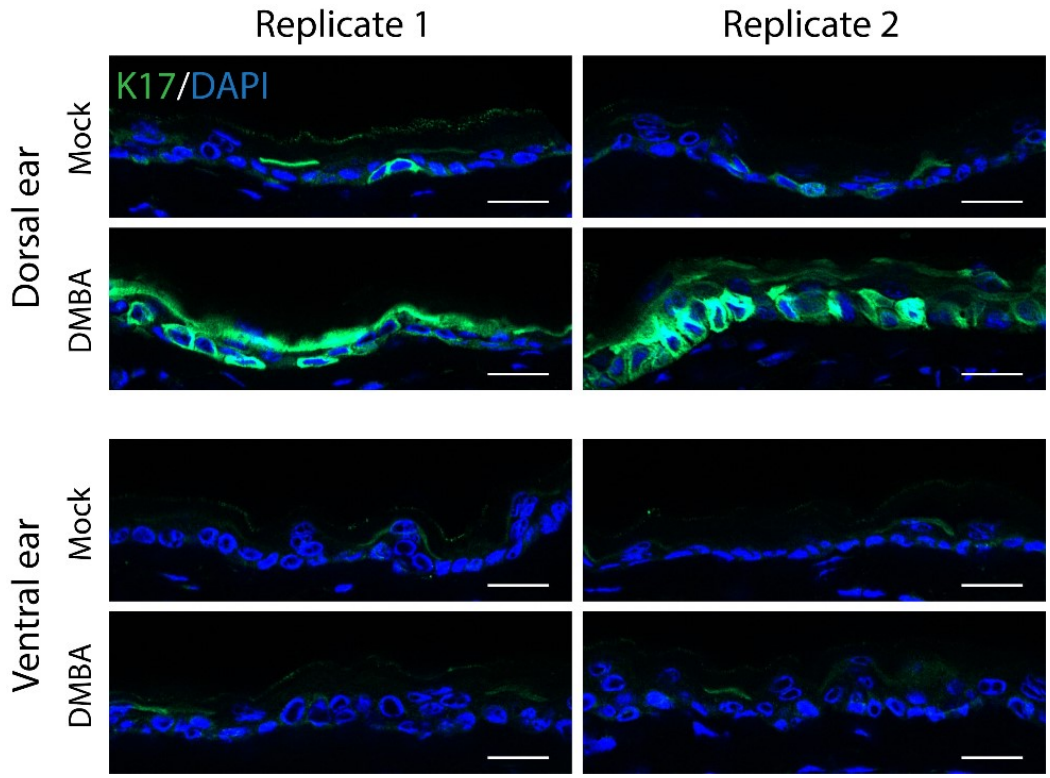

Figure 6. K17 induction *in vivo* upon DNA damage insult. 2 months old *Krt17*$^{+/-}$or *Krt17* $^{+/+}$ female mice were treated topically on the dorsal ear surface (20 µg of DMBA dissolved in acetone on right ear, acetone only (mock) on left ear) two times, 24 hrs apart. Ear tissue was harvested 1 hr after the second treatment and embedded in OCT and frozen. 8 µm tissue cryosections were immunostained for K17 (indirect immunofluorescence). K17 is induced the interfollicular epidermis of the dorsal ear but not the ventral side upon treatment of DMBA. Images are representative of multiple tissue sections from two distinct biological replicates. All scale bars = 20 µm. Figure from R. Hobbs, 2017.

Nuclear K17 co-localizes with γH2AX

As described previously, indirect immunofluorescence microscopy data showed a correlation between the induction of K17 and treatment with DMBA. With the knowledge of the presence of nuclear K17, we sought out whether nuclear K17 may associate with γH2AX, a well-known DDR effector that resides within the nucleus and is known to be induced upon DNA damage, such as DMBA treatment. To do this, normal human epidermal keratinocytes (NHEKS) were first co-stained by indirect immunofluorescence with antibodies γH2AX and

PML, which has been reported to closely associate with K17 in tumor epithelial keratinocytes (unpublished observations, Coulombe laboratory) (Figure 7a). Building upon this finding, HeLa cells in culture were treated with 40 nM of LMB for 3 ½ hours, 100 ng/μL of TNF-α for 3 hours, and 200 nM of TPA for 3 hours to induce the formation of intranuclear K17 punctae. Utilizing indirect immunofluorescence microscopy, images stained for both nuclear K17 and γH2AX showed co-localization between the two proteins (Figure 7b), suggesting that nuclear K17 may physically interact with γH2AX to regulate the DDR response.

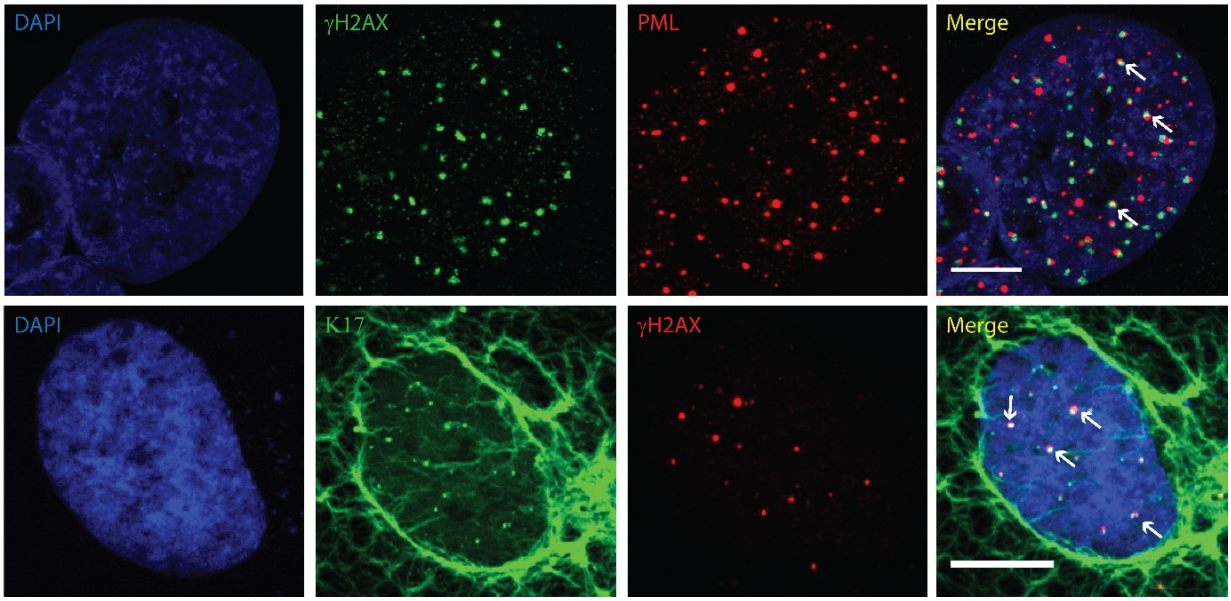

Figure 7. Nuclear K17 juxtaposed with γH2AX and PML bodies. (a) Normal Human Epidermal Keratinocytes (NHEK) were treated with 1 μg of DMBA (12 hrs) and immunostained for γH2AX and PML. The images represent distinct γH2AX foci its co-localization to respective nuclear proteins (arrows). (b) LMB-treated HeLa cells immunostained for K17 and γH2AX. The merge image to the right highlight K17-positive nuclear punctae (arrows) and co-localization with γH2AX is observed. All scale bars = 10 μm.

Genetic ablation of K17 significantly attenuates the DDR response

To further investigate whether K17 plays a pivotal role in the DDR response, the human epidermoid carcinoma cell line A431 (Adhikary et al., 2013) was utilized to address this

overarching question. CRISPR/Cas9 genome editing technology was used to genetically ablate *Krt17*, resulting in A431 *KRT17* $^{-/-}$ cells (Hobbs et al., 2015). Parental cells and *KRT17* null cells were treated in parallel with either DMBA (for 12 hours; Ganesan et al., 2013) or H_2O_2 (for 30 min; Driessens et al., 2009) and analyzed using indirect immunofluorescence. As illustrated in Figure 8, the latter revealed an induction of γH2AX levels in parental cells, but a significant attenuation of γH2AX foci in null cells ($p<0.005$). This, along with previous data showing an inducible nature of K17 upon damage, suggests K17 is indeed required for the induction of the DDR (Figure 8). Again, to further corroborate this finding, a similar experiment was conducted *in vivo*, where ear tissue sections from two-month-old female mice (*KRT17* $^{+/-}$ or *Krt17* $^{-/-}$) were immunostained for γH2AX after treatment with DMBA or acetone control. Correlating with the *in vitro* results, γH2AX is seen to be more upregulated when K17 is present (Figure 9).

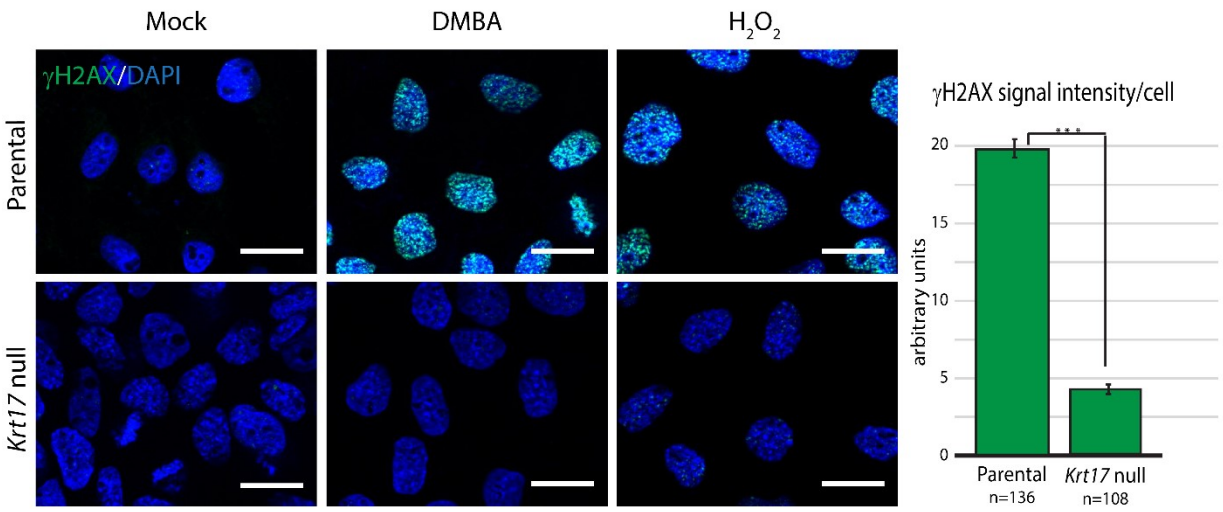

Figure 8. K17 dependence on γH2AX induction. A431 skin tumor keratinocytes (parental= K17 expressing; *KER17* null = CIRSPIR/Cas9 mediated genetic ablation of K17) were seeded on glass cover slips, treated with 1 µg of DMBA (12 hrs) or 100 µM H_2O_2 (30 min), fixed with 4 % paraformaldehyde, 0.2% triton permeabilization, and indirect immunostained for γH2AX. All scale bars = 20 µm. Signal intensity per cell is quantified in the graph at the right using ImageJ and Excel. n= number of cells counted. *$p<0.005$ (Student's t-test).

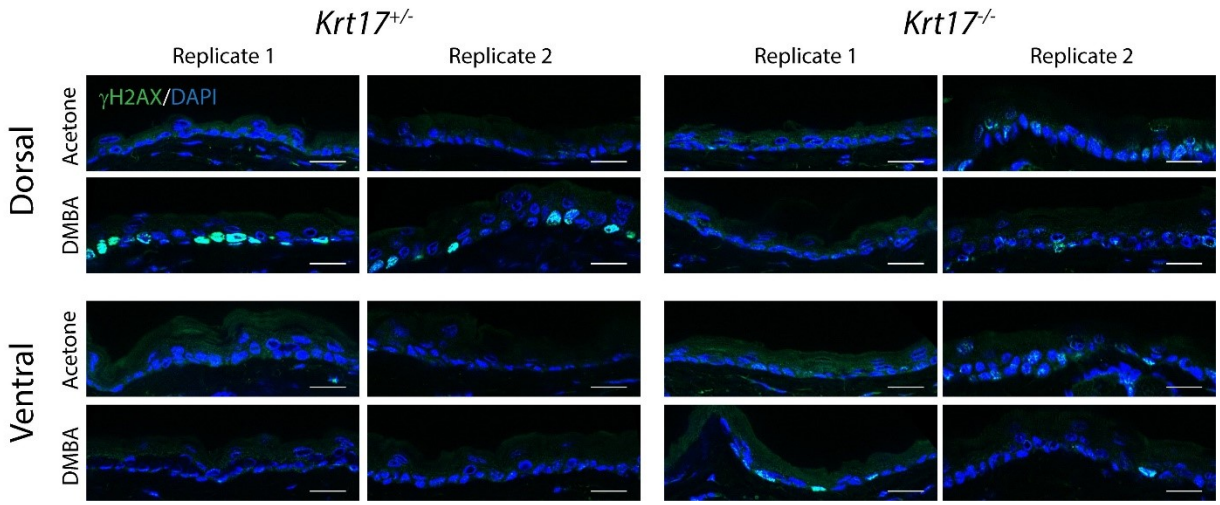

Figure 9. K17 dependence on γH2AX induction *in vivo*. 2 months old *Krt17*$^{+/-}$or *Krt17* $^{-/-}$ female mice were treated topically on the dorsal ear surface (20 µg of DMBA dissolved in acetone on right ear, acetone only (mock) on left ear) two times, 24 hrs apart. Ear tissues were harvested 1 hr after the second treatment and embedded in OCT. 8 µm tissue sections were immune stained for γH2AX. γH2AX is induced in the interfollicular epidermis of the dorsal ear but not the ventral side upon treatment of DMBA. Induction of γH2AX.is attenuated in the absence of *Krt17*. Images are representative of multiple tissue sections from two distinct biological replicates. All scale bars = 20 µm.

Next, we sought to expand our assessment of the K17-dependence of the entire DDR pathway by examining the status of a broad range of DDR markers. Again, parental and null keratinocytes were treated with either DMBA or H_2O_2 for the indicated times and harvested for analysis via western immunoblotting. At the protein level, the occurrence of DNA damage is evident under these treatment conditions, as demonstrated by immunoblotting for γH2AX (Figure 10). *Krt17*$^{-/-}$, however, showed an attenuation of γH2AX induction, parallel with the microscopy data observed earlier. The induction of other DDR markers, such as p-BRCA1, pATM, pATR, pCHK1, pCHK2, and p-p53 all displayed an increase in band intensity when treated with DNA damaging agents when K17 is present. Again, these same DDR markers displayed a visually distinct lower band intensity in the *Krt17* null setting (Figure 10),

suggesting attenuation of the DDR. Taken together, K17 appears to not only impact γH2AX levels in the DDR, but a plethora of DDR proteins in the signaling cascade.

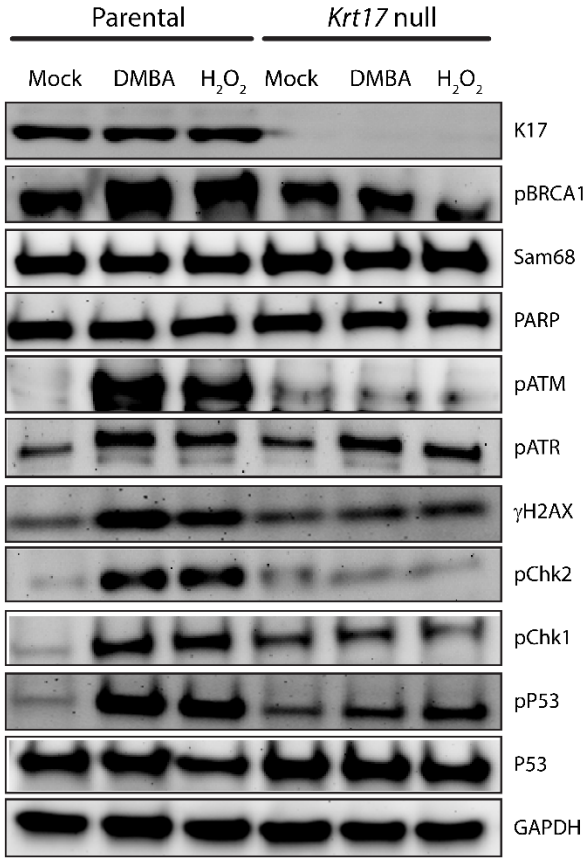

Figure 10. K17 impacts the canonical DNA damage response pathway upon insult. Immunoblotting analysis of total protein extracts prepared from A431 skin keratinocytes subjected to DNA damage agents showed that the DDR is activated in parental cells by DMBA or H2O2, as indicated by increased levels of phospho-ATM, phospho-ATR, phospho-Chk1, phospho-Chk2, phosphor-p53 and γH2AX. By comparison, cells lacking K17 showed attenuation of the DDR.

Nuclear-localized K17 is required for the DNA Damage Response

To further dissect the impact K17 plays in the DNA damage response, a re-expression strategy was carried out to investigate whether transfection of a CMV-promoter-driven, wildtype K17-encoding GFP plasmid (Hobbs et al., 2015) is sufficient to restore the DNA damage response in *Krt17*$^{-/-}$ A431 cells. Using indirect immunofluorescence microscopy, levels of γH2AX in *Krt17* null cells that successfully re-expressed wildtype K17 were compared to

neighboring untransfected cells. Quantitatively, there is a significant difference between the two, whereby successfully transfected cells displayed much higher induction of γH2AX when compared to "control" untransfected cells (in the same dish), indicating re-expression of *Krt17* is sufficient to rescue the DNA damage response (Figure 10). Taking a step further, cultures of *Krt17* null A431 keratinocytes were transfected with the ΔNLS variant of GFP-*Krt17*, or wildtype *GFP-Krt14*, a type I keratin related in K17 in primary structure. Both ΔNLS GFP-*Krt17* and WT GFP-*Krt14* were unable to rescue the induction of γH2AX, suggesting that that the nuclear-localized from of K17, specifically, is needed to foster a normal DNA damage response (wildtype K17 vs K17-ΔNLS, p=0.024; wildtype K17 vs wildtype K14, p=0.034; WT-K17 vs null, p=0.004. Taken together, this, along with the data presented above regarding K17's interaction with γH2AX in the nucleus, hints at a "sensing" role K17 may play in promoting an "upregulation" of the DNA damage response.

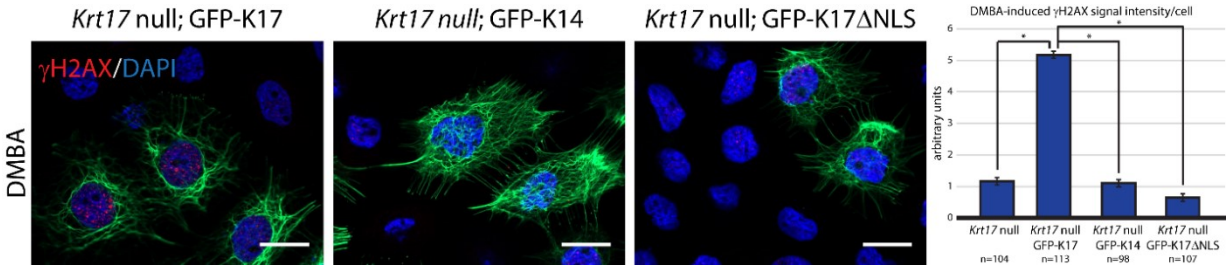

Figure 11. Re-expression with K17, but not K14 or K17 lacking a nuclear localization sequence, restores the DDR in A431 tumor keratinocytes. *Krt17* null A431 skin tumor keratinocytes were seeded on glass cover slips and subsequently transiently transfected with either GFP-K17, GFP-K14, or GFP-K17ΔNLS, treated with 1 μg of DMBA (12 hr), fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and immunostained for γH2AX. Expression of GFP-K17, but not GFP-K14 or GFP-K17ΔNLS, restored the ability of *Krt17* null cells to induce γH2AX expression following DMBA treatment. Signal intensity of γH2AX per cell expressing each construct is quantified in the graph to the right using ImageJ and Excel. All scale bars = 20 μm. n= number of cells counted. *p<0.05 (Student's t-test).

K17 sensitizes DNA to more strand breaks

The idea of K17 playing a sensing role in the DDR by altering chromatin architecture is plausible, since it has already been seen to bind transcription factors such as AIRE (which has been reported to occur at double-strand DNA breaks; Anderson & Su, 2016) and the p65 subunit of NF-kappaB (Hobbs et al., 2015). As already mentioned above, recent literature reports on the occurrence of crosstalk between DNA damage and the organization of the chromatin (Sulli & Di Micco & d'Adda di Fagagna, 2012), as well as a significant role for K17 in modulating the onset of tumorigenesis and pro-inflammatory expression *in vivo* (DePianto et al., 2010; Hobbs et al., 2015) and ex vivo (Chung et al., 2015). Together with the data reported so far in this thesis, this body of evidence indeed suggests that the presence of nuclear K17 may play a role in sensing DNA damage by possibly altering the chromatin structure in a manner that results in higher susceptibility to external insults. To address this question, we next turned to the comet assay, which has been extensively utilized as an indicator for both single-strand and double-strand DNA breaks (Fairbaim, Olive & O'Neill, 1995). A431 skin keratinocytes (*Krt17*$^{+/+}$or *Krt17* $^{-/-}$) were treated with DMBA (12 hrs) and H_2O_2 (30 min), as described above, and cells were next suspended and plated on an LMN agarose slide and subjected to electrophoresis (see Materials and Methods). As observed in all four biological replicates (n=4), the parental cells treated with either DMBA or H_2O_2 showed significant longer tail moments compared to the null (p=0.002), indicative of a higher extent of DNA fragmentation (Figure 11). This finding is in accord with the hypothesis described previously, where K17's sensing mechanism in the DDR is sensitizing the chromatin to fragmentation. To further substantiate these findings, ionizing radiation (IR) experiments were carried out to investigate if K17 does really impact the level of DNA breakage, since IR is a very reliable source of

causing double strand breaks (DSB), thereby fragmenting DNA. Doses of 2 Gy and 20 Gy were chosen for indirect immunofluorescence microscopy and comet assays, respectively. As shown below, (n=3 biological replicates) there is a K17 dependence on DNA breakage indicated by a higher γH2AX signal intensity, which also shows that K17 dependence on DNA breakage is correlated with a more robust DNA damage response. (Figure 12). The alkaline comet assays treated with irradiation also shows K17 dependence on DNA fragmentation, correlating with previous observations with treatment with DMBA and H_2O_2 (Figure 13).

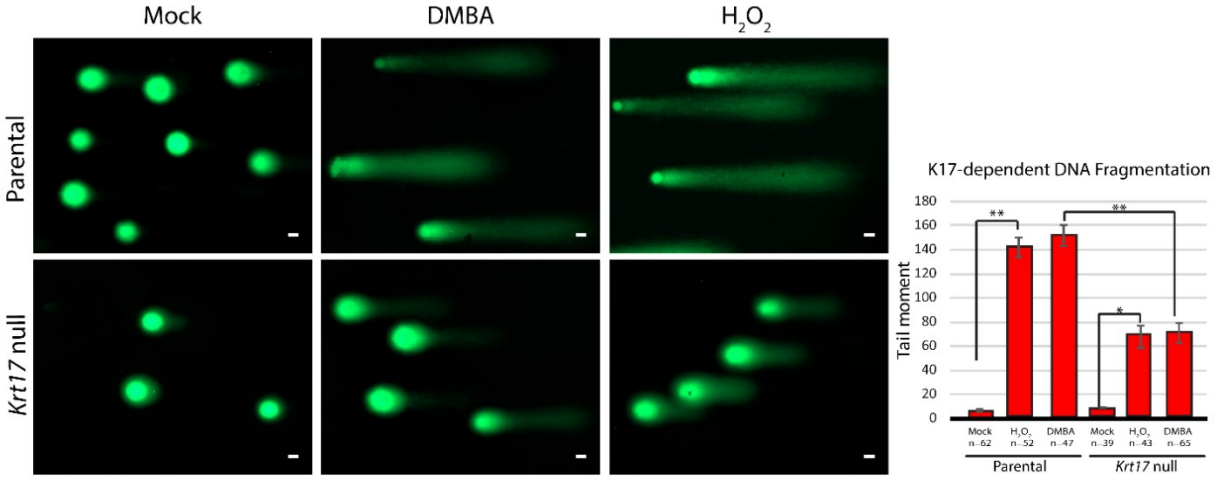

Figure 12. Depletion of K17 attenuates DNA breakage. A431 skin tumor keratinocytes were subjected to DNA damaging agents and processed immediately post DNA damage for an alkaline comet assay to assess DNA fragmentation. Longer tail moments are observed in parental cells when exposed to various insults, which are substantially reduced in the absence of K17. Treatment conditions are as the same as above. All scale bars = 20μm

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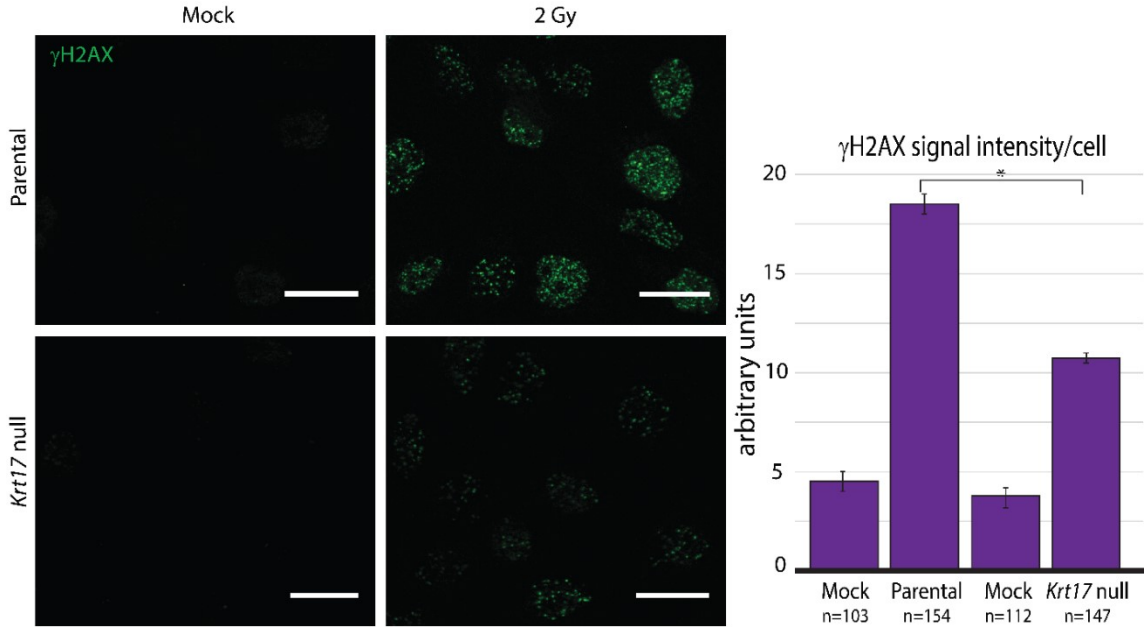

Figure 13. K17 dependence on γH2AX induction under ionizing radiation A431 skin tumor keratinocytes (parental= K17 expressing; *Krt17* null = CIRSPIR/Cas9 mediated genetic ablation of K17) were seeded on glass cover slips, treated with 2 Gy ionizing radiation, fixed with 4 % paraformaldehyde, 0.2% triton permeabilization, and indirect immunostained for γH2AX. All scale bars = 20 μm. Signal intensity per cell is quantified in the graph at the right using ImageJ and Excel. n= number of cells counted. *p<0.005 (Student's t-test).

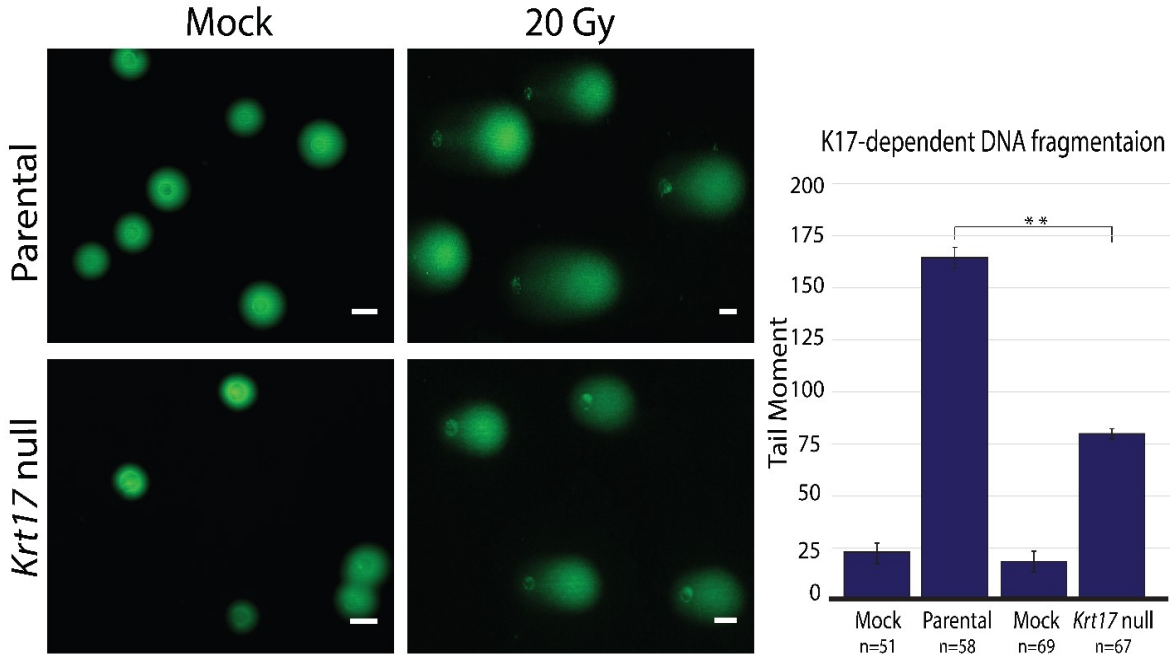

Figure 14. Depletion of K17 attenuates DNA breakage under ionizing radiation. A431 skin tumor keratinocytes were subjected to 20 Gy ionizing radiation and processed for an alkaline comet assay to assess DNA fragmentation. Longer tail moments are observed in parental cells when exposed to various insults, which are substantially reduced in the absence of K17. Treatment conditions are as the same as above. All scale bars = 20 μm. Tail moment lengths per cell is quantified in the graph at the right using ImageJ and Excel. n= number of cells counted. *p<0.005 (Student's t-test).

The use of a micrococcal nuclease digestion assay (MNase) is well documented in the literature as a means to assess general chromatin architecture (Yamasaki et al., 2007; Wu et al., 2011; Ziv et al., 2006). Micrococcal nuclease is an enzyme that cleaves transcriptionally active sites, thus, a euchromatin state will be more prone towards degradation by this enzyme. The cleaved fragments are subsequently subjected to agarose gel electrophoresis to visualize the banding patterns. This assay has been utilized in countless investigations to address questions such as histone modifications and its ability to impact chromatin structure (Wu et al., 2015).

To test whether K17 actually impacts chromatin organization, parental and *Krt17* null A431 skin keratinocytes were permeabilized with lysolecithin, allowing micrococcal nuclease to translocate into the nucleus and cleave at transcriptionally active sites on the DNA. Ethylenediaminetetraacetic acid (EDTA) and proteinase K (Monico et al., 2017) was added in a stop solution to halt genome digestion. The micrococcal nuclease enzyme was added for increasing time periods to assess the state of chromatic packing (see Materials and Methods). Here, we report that K17 impacts the extent of MNase-dependent DNA fragmentation, whereas cellular DNA was more susceptible to MNase digestion in its presence (Figure 13b). To complement these findings, a distinct DNA fragmentation assay was performed following exposure to ionizing radiation (IR) as a direct inducer of double strand DNA breaks. The rationale here is to probe for a potential interdependency between K17's role in sensing DNA damage and its potential role in influencing chromosome architecture. To first assess the paradigm of IR induced fragmentation, parental cells and nulls were subjected to 2 Gy or 20 Gy of IR, as previously described (Puck & Markus, 1956), and lysed to extract DNA for the purpose of gel electrophoresis. Qualitatively, the results obtained paralleled the data observed in the MNase experiments, with the presence of K17 significantly increasing the extent of DNA fragmentation (Figure 13a). Taken together, these findings suggest that the presence of K17 alter chromatin architecture so as to promote a euchromatin, looser state, thereby sensitizing DNA towards further degradation with MNase and ionizing radiation.

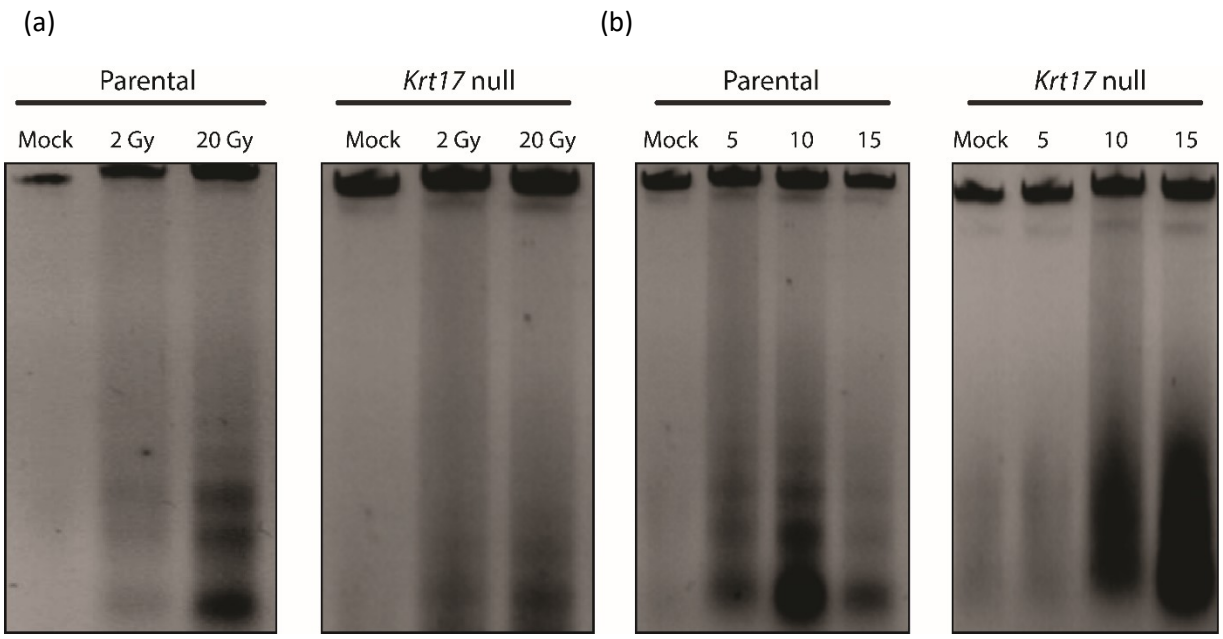

Figure 15. K17 leads to higher levels of DNA fragmentation. A431 skin tumor keratinocytes were subjected to a range of ionizing radiation (IR) or various concentration of micrococcal nuclease. DNA was isolated and ran on an agarose gel. Lower molecular weight bands were more prominent in the parental cells, where the absence of K17 appeared to be more resistant to DNA fragmentation.

Discussion

The research project described in this thesis uncovers a novel role of K17 in specifying the sensitivity and/or the repair response for three different DNA damage paradigms, and as such has significant implications for the impact of K17 at the time of tumor initiation *in vivo*.

Stemming from the extensively reported observation that K17 is induced in a variety of stress/inflammatory settings, and coupled with the knowledge that K17 directly impacts the course of tumorigenesis through a number of mechanisms, we sought to investigate whether K17 plays a role in the initiation stage of tumorigenesis, through subjecting cultured cell lines to acute DNA damage with three different types of insults, as well as *in vivo* experiments

involving mouse skin. This acute model is a logical choice since it draws upon the inducible nature of K17 and provides a parallel analysis of the inducible nature of DDR markers as well, also providing a time sensitive, finely controlled approach to inducing DNA damage. Our findings indicate that treatment with the mutagen DMBA and the oxidative species H_2O_2 elicits an upregulation of K17 concurrent with a robust stimulation of the DNA damage response, as indicated by γH2AX staining, in skin keratinocytes isolated from mice tissue and seeded in primary culture.

This piece of evidence was complemented by our ability to successfully visualize co-localization of nuclear K17 and γH2AX, suggesting a potential physical association. However, the latter experiment was only carried out once (n=1 biological replicate) due to the fact that Hela cells are very sensitive towards LMB/TNF-α/TPA treatment and can result in cell death. Fortunately, recent mass-spectrometry data revealed a physical association between γH2AX and K17, further substantiating its possible interaction (unpublished observations, Justin Jacob, Coulombe laboratory). To validate this, co-immunoprecipitation could be carried out in the future to visualize molecular binding between the two proteins. Probing for other DDR markers is a plausible idea as well, and it would be interesting to see if K17 physically interacts with one or more effectors that play a role in sensing DNA damage, ahead of the repair response per se. ATM, for example, represents an interesting candidate, since this protein has been reported in literature to physically interact with γH2AX (Wang et al., 2016). Other upstream molecules in the DNA damage response are viable options as well; it would only substantiate our hypothesis if nuclear K17 is seen to physically associate with a plethora of DDR markers, suggesting K17 is a critical component to the DNA damage response, from sensing to actual repair.

In order to dissect the role that K17 plays in the DNA damage response, human tumor keratinocytes expressing K17 or lacking K17 were subjected to three types of insults: DMBA, H_2O_2, and IR. Analyses showed significant attenuation of the DDR in cells lacking K17, as indicated by lower γH2AX intensity. Extensive experimentation was carried out to assess the depth of K17' impact on the DDR. Western blot analysis displayed a significant attenuation of DDR across a broad range of relevant markers when K17 is not present. Closer examination of the immunoblot data suggest that K17 is only impacting the damage proteins on a post-translational level, since only the phospho-levels of markers being induced, not total protein levels, are typically affected. This result is concurrent with recent literature, where the DNA damage response is a transient event, where phosphorylating markers is a more effective means of signal transduction (Sulli, Di Micco & di Fagagna, 2012). Looking carefully, it is interesting to note that p-ATR is not dramatically different between the two genotypes, whereas p-ATM is. This suggests the possibility where nuclear K17 may not just play a sensing role following DNA damage, but a specific pathway in the DNA damage response. Thus, further experiments such as co-IP probing for ATM would be a worthy experiment. This differential impact of K17 loff on ATM vs. ATR activation also raises the question of where and when is K17 performing its sensing abilities. Since the difference in the response to damage may be at the p-ATM/p-ATR level, it suggests that K17 impacts the sensing at this level or higher upstream. To address this question, laser ablation experiments can be conducted to visualize the dynamics of DDR protein recruitment (Sun et al., 2016) to better understand if K17 is impacting a certain protein recruitment to sites of damage, or the activation of that protein, or both.

To test if re-expressing *Krt17* in human keratinocytes could rescue the DNA damage response, WT GFP-*Krt17* was transiently transfected into A431 keratinocytes under the

treatment of either DMBA or H_2O_2. Detailed analysis with thorough quantification indicated a restoration of the DDR to a highly significant degree in *Krt17* $^{-/-}$ keratinocytes transfected with WT GFP-*Krt17*, as indicated by a very high γH2AX intensity. Because the transfection protocol used is transient and is effective for a subset of cells in the dish, one could readily that cells displayed drastic differences in γH2AX intensity depending on whether they expressed WT GFP-*Krt17* or not, providing a powerful internal reference. By contrast, transient transfection with related type I keratin, WT GFP-*Krt14*, or a K17 variant with compromised nuclear import, ΔNLS GFP-*Krt17*, resulted in no significant change in the rescue of the damage response.

Consistent with the data already discussed above, K17 appears to impact most of the proteins involved in DNA damage by influencing the level of phosphorylation across different paradigms of DNA insults. However, it is worthwhile to note that the induction of p-ATR is not dependent on K17, hinting at the possibly that K17 may play a role in DDR through an ATM specific signaling event. Because the evidence showed K17 dependence in a plethora of proteins involved in DDR signaling, we hypothesized that K17 may be affecting the DDR at an even up-stream level of the damage response. This lead us to believe that K17 plays a sensing role in the damage response, consequently altering chromosome architecture, allowing DNA to be more susceptible towards external insults, resulting in enhanced DNA breakage. From this, we carried out a variety of experiments to determine this keratin's ability to influence the level of DNA fragmentation. Tail-moments observed from comet assays were significantly longer in skin keratinocytes expressing *Krt17* and were attenuated in the absence of K17. This piece of evidence solidified our confidence of this keratin's ability to increase the levels of DNA breakage, leading us to conduct the MNase and DNA fragmentation assays for further

verification. Both of the assays conducted showed an increased level of DNA fragmentation when K17 was present.

The findings reported in this thesis, when coupled with the body of work produced in the Coulombe laboratory and others, underscore the significance of K17 in a number of disease settings. In summary, we were able to arguably tether the realm of K17's role in the DNA damage response to its ability to orchestrate chromatin architecture, ultimately determining the susceptibility of chromatin to fragmentation. These findings, taken together with previous experiments, suggests that K17 may play a role in sensing DNA damage, and therefore in the initiation the DNA damage response, by promoting the organization of nuclear DNA into a euchromatin state, thereby sensitizing it towards fragmentation. This, in turn, would result in an even higher activation of the DDR pathway. However, key questions remain: What is the purpose of K17 sensing or impact on the DDR as part of its inherent homeostatic functions in the human body? And how might this role be involved early on during tumorigenesis? Ultimately, cell fate experiments must be carried out in order to gain insight into these defining questions.

To further expand on our knowledge of K17 involvement in DNA damage, future efforts could be directed as followed: i) ChIP-seq analyses may provide insight as to where K17 might be binding to the chromatin and what histone modifications, for instance, may be involved in its ability to alter chromosome architecture; ii) a more extensive investigation of K17 and its role in DNA repair by conducting *in vitro* non-homologous end joining (NHEJ) and homologous repair (HR) assays to elucidate if K17 is involved in a specific repair pathway; iii) perform additional assays that may further substantiate K17's role in impacting chromatin organization in the nucleus, such a fluorescent in situ hybridization (FISH); iv) repeat the experiments

performed in this thesis project in murine models, given the new availability of the a K17 ΔNLS knock-in mouse model to assess DNA damage; and iv) subject *WT* and *Krt17* null mice to the two-step (DMBA/TPA) carcinogenesis protocol (need ref), with a particular focus on papilloma formation, to assess the role K17 has in the setting of tumor initiation *in vivo*. Completing those experiments would extend the findings of the current project, and validate (or not) the tentative conclusion that nuclear-localized K17 acts impact the extent of DNA damage as well as the response to it response primarily by altering chromatin structure and possibly by being part of the DNA damage sensing apparatus. The novel discovery of a role for K17 in DDR, coupled with its known role as a regulator of inflammation, suggests that this intermediate filament protein, when translocated into the nucleus, is able to promote these enabling characteristics during the initiation stage of tumorigenesis.

MATERIALS AND METHODS

Cell lines

Mouse epidermal keratinocytes were isolated for primary culture or immortalized as described previously. Parental A431 and HeLa cell lines (American Type Culture Collection) were confirmed to be mycoplasma free. The generation of A431 cells stably expressing shRNA targeting *KRT17* was conducted by CRISPR-Cas9-mediated genome engineering. Specifically, a target sequence in the first exon of human *KRT17* (5'-GGCTCCTCCGGCCTGGGGGGCGG-3'(Pam motif underlined)) was chosen, and a 20 nucleotide guide sequence (5'-GGCTCCTCCGGCCTGGGGGG-3') was cloned into the BbsI site of pX458 (pSpCas9(BB)-2A-GFP, which is obtained from AddGene (plasmid 48138) according to the cloning protocol. For sequencing, the *KRT17* regions targeted in the knockout cell line were PCR amplified and cloned into the pJET1.2/blunt cloning vector using the CloneJET PCR cloning kit (Life Technologies, K1231). Sequencing results showed a frameshift and premature stop codon formation for both alleles of *KRT17.*

Mouse Models

All experimental protocols involving mice were approved by the Johns Hopkins Institutional Animal Care and Use Committee. Genotyping for the $Krt17^{+/+}$ and $Krt17^{-/-}$ alleles were conducted according to national protocols (National Cancer Institute Repository, strain 01XT3; McGowan et al., 2002). FVB/N $Krt17^{+/-}$ and FVB/N $Krt17^{-/-}$ mice were utilized in this study.

Tissue Collection

Ear tissues were harvested from five to six mice (2 month of age) for each genotype (*Krt17*$^{+/-}$ and *Krt17*$^{-/-}$) after a double topical DMBA (20 μg) or acetone treatment, 24 hours apart, then harvested one hour later after the second treatment. All tissue sections were quickly embedded in Tissue-Tec OCT compound (Sakura Finetek, Torrance, CA, USA) and stored at -20°C prior to sectioning. Tissue sections were cut at 8 μm in thickness and subsequently subjected to indirect immunofluorescent staining.

Antibodies, reagents and plasmid constructs

The primary antibodies used in these studies included rabbit polyclonal antibodies against Krt17 (McGowan, 1998), Sam 68 (Santa Cruz Biotechnology sc-333, Dallas, TX, USA); rabbit monoclonal antibodies against histone H3 (Cell Signaling #9715, Danvers, MA, USA), γH2AX (serine 139) (Cell Signaling #9718, Danvers, MA, USA), p53 (Cell Signaling #2527, Danvers, MA, USA), phospho-BRACA1 (Serine 1524) (Cell Signaling #9009, Danvers MA, USA), phospho-ATR (Serine 428) (Cell Signaling #2853, Danvers MA, USA), phosphor-ATM (serine 1981) (Cell Signaling #5883, Danvers, MA, USA), PARP (Cell Signaling #9532, Danvers, MA, USA), phospho-Chk2 (threonine 68) (Cell Signaling #2197, Danvers, MA, USA), and phospho-Chk1 (serine 345) (Cell Signaling #2341, Danvers, MA, USA); mouse monoclonal antibody against phospho-p53 (serine 15) (Cell Signaling #9286, Danvers, MA, USA); normal rabbit IgG (Santa Cruz Biotechnology sc-2027, Dallas, TX, USA); mouse polyclonal antibodies against GAPDH (Santa Cruz Biotechnology sc-365062, Dallas, TX, USA). Secondary antibodies included ones conjugated to Alexa Flour 488 (Abcam, Cambridge, UK), Alexa Flour 647 (Abcam, Cambridge, UK) Alexa Flour 594 (Abcam, Cambridge, UK) for indirect immunofluorescence and horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies (Sigma-Aldrich, St. Louis, MO, USA) for

chemiluminescence immunoblotting. 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Roche 10236276001, Mannheim, Germany) was used as a marker for indirect immunofluorescence. All antibodies were used according to the manufacture's recommendation. TPA (Sigma, P1585) was dissolved in DMSO (for cell treatments) and used at 200 nM or 25 ng/ml working concentration, respectively. LMB (Sigma, L.2913) was dissolved in 70% methanol and used at a 40.7 nM working concentration. The *Krt17* plasmid were transiently transfected into A431 cells using FuGENE HD (Promega, E2311) at a 1:3 (DNA amount;FuGENE volume) ratio according to the manufacturer's instructions. Micrococcal Nuclease 300 U/μL was obtained from Thermo Scientific #EN0181, Waltham, MA, USA.

γ-Irradiation

The γ-irradiation on A431 keratinocytes was performed using a ^{137}Caesium source (dose rate 4 Gy/min).

Western Blotting, Indirect Immunofluorescence and Microscopy

Protein lysates for immunoblotting were prepared in urea sample buffer (8 M deionized urea, 0.5% SDS, 30 mM Tris, pH 6.8, 5% glycerol and 5% β-mercaptoethanol) after collecting whole cell lysates from cultured cells. All samples were sheared using progressively finger-gauged needles (22 1/2, 25 1/2, and 26 ½) and subjected to a Bradford assay and processed. All samples were loaded at 10 μg of protein per lane for immunoblotting. Samples were separated using either 10% or 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to 0.45μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) via a trans-blot turbo transfer system (Bio-Rad). Blots were blocked in 5% milk in TBST- T (w/v)

for >1hr at room temperature (21°C) and subsequently incubated overnight at 4°C in their appropriate antibodies. Secondary antibodies were applied for 1hr at room temperature (21°C). Blots were developed using either ECL Select developing solution (GE Healthcare, Pittsburgh, PA, USA) or SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) and imaged using a FluorChem Q system (ProteinSimple, San Jose, CA, USA).

For indirect immunofluorescence analysis, frozen sections of the aforementioned tissues were blocked for 1hr at room temperature (21°C) in 5% normal goat serum and 1X PBS. Primary antibodies were applied overnight at 4°C. For cultured cells, they were needed on collagen coated glass cover slips (1:100 ratio of collagen and 0.2% acetic acid), then fixed and permeabilize with 4% paraformaldehyde and 0.2% TritonX, respectively. Primary antibodies were applied subsequently followed with secondary antibodies, with 30 minute incubations in between. Cover slips were mounted onto slides with FluoroSave ^{TM} reagent (345789-20ML, Calbiochem) and dried overnight. Both individual cells and tissue sections were imaged using a Zeiss fluorescence microscope with Apotome attachment (Zeiss, Oberkochem, German). Images for the same marker, across all samples and genotype were acquired at the same exposure, pixel range, gamma values and objective using Zen software. All images were consistently adjusted for brightness and contrast and appropriately cropped for presentation purposes using ImageJ software.

Alkaline Comet Assays

Comet assays were conducted using the Comet Assay Kit (Trevigen) following the manufacturer's instructions. A431 keratinocytes were mock treated or treated with DMBA, H_2O_2, or IR, with the indicated doses. Cells were collected and washed once with PBS, and 3 X 10^5 cells were combined with 1% molten LMAgarose at 37 degrees Celsius at a ratio of 1:10

(v/v) and immediately pipetted onto slides. Slides were then immersed in pre-chilled lysis buffer to lyse cells, followed by alkaline unwinding of chromatin. Alkaline electrophoresis of gelled slides was conducted at 30V at 4 degrees Celsius for 30 minutes. The DNA was visualized by SYBR gold staining, and images were taken with a Zeiss fluorescence microscope with Apotome attachment, then analyzed by CometScore software (Tri-Tek, Sumerduck, Virginia).

Micrococcal nucleus digestion assay

MNase digestion assay was carried out as previously described. Briefly, A431 keratinocytes were washed with cold PBS and permeabilized with permeabilization solution 1 (150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) for 5 minutes. Solution was aspirated and treated with 0.05% lysolecithin for 1 minute. The solution was then again aspirated and permeabilization solution 2 (150 mM sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM $CaCl_2$) containing indicated concentrations of MNase were incubated at different time points. The reaction was stopped by adding stop solution buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 20 mM EDTA, 1% SDS, 0.6 mg/mL protinase K) and lysis dilution buffer (150 mM NaCl, 5 mM EDTA). Suspended homogenous solution was incubated overnight at 37 degrees Celsius with 4 uL of RNAase and TE buffer, pH 8.0. Phenol-chloroform extraction and ethanol precipitation was utilized to precipitate fragmented DNA, then subsequently separated by agarose gel electrophoresis, stained by ethidium bromide.

Image Quantification, Graphical Analysis and Description of Statistical Methods

Signal intensity quantification for indirect immunofluorescence images were performed using ImageJ software. The calculations were based on quantifying each individual cell within each field at 40X magnification, where the γH2AX signal intensity for each cell is measured (n=3 biological replicates). Background intensity was subtracted from each γH2AX signal intensity to be used as baselines. Graphs accompanying IF images represent the average overall arbitrary units of γH2AX levels in individual cell nuclei that were subjected to various DNA damaging agents. Error bars in quantification of immunofluorescence images represent technical error across multiple replicate images. All P-values were generated using a Student's t-test with two-sided distribution and equal variance via Microsoft Excel. * denotes a P-value of <0.05; ** denotes a P-value of <0.02. Quantification for alkaline comet assays were performed using CometScore software. The calculations were based on measuring tail lengths for each individual cell within the field of microscopy, at 10X magnification. Graphs accompanying alkaline comet assays represent the average tail lengths between each treatment conditions and its respective genotype. Error bars in quantification of immunofluorescence images represent technical error across multiple replicate images. All P-values were generated using a Student's t-test with two-sided distribution and equal variance via Microsoft Excel. * denotes a P-value of <0.05; ** denotes a P-value of <0.02.

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JOSHUA HSU

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EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD **Expected May 2017**
Master of Science, (Sc,M.)
Concentration: **Biochemistry & Molecular Biology (Reproductive and Cancer Biology)**
GPA: 3.9

Drew University, Madison, NJ **May 2015**
Bachelor of Arts, (B.A.)
Major: Biochemistry & Molecular Biology
Minor: Public Health
GPA: 3.5

RESEARCH EXPERIENCE

Graduate Researcher, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
PI: Dr. Pierre Coulombe, Ph.D. **March 2016-Present**

- Original thesis research investigating the interplay between Keratin 17 and the DNA damage response to elucidate the keratin's functional role in oncogenesis and chronic diseases. So far, not only does K17 play a role in DNA damage susceptibility, it also contains epigenetic qualities by shuttling into the nucleus and relaxing chromatin to express certain genes.
- K17 dependence on DNA damage was evaluated with western blots derived from CRISPR-Cas generated KO cell lines and mice colonies. Additional techniques such as comet assays, immunofluorescent staining was utilized to compliment the data. MNase hypersensitivity assays and CHIP sequencing were utilized to elucidate the role of keratin 17 on chromatin organization.

Graduate Research Assistant, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
PI: Dr. Phillip Jordan, Ph.D. **Sep 2015-Feb 2016**

- Assisted in the investigation of Aurora kinases/SMC proteins and their role in carcinogenesis and infertility by performing westerns and chromosome spreads needed for fluorescence microscopy.
- Performed seminiferous tubule squashes for the analysis of male chromosome dysregulation
- Histological analysis of seminiferous tubules and preantral follicles.

Undergraduate Research Assistant, Drew University RISE, Madison, NJ **Oct 2012-June 2015**
Advisor: Dr. Ronald J. Doll, Ph.D.

- Drug discovery project: Synthesized and purified novel benzimidazole and quinoline class compounds via standard organic chemistry techniques. Compounds were characterized by LC/MS, GC/MS, and NMR. They were then tested for reactivation of mutant p53 in human tumor cells lines via westerns and cell viability assays.
- Established Drug Metabolism and Pharmacokinetics (DMPK) of synthesized compounds to determine drug absorption, metabolism, permeability and toxicity.
- Successfully synthesized analogs of Kevetrin™, an anti-cancer agent in phase 1 clinical trials.

Clinical Research Assistant, Weill Cornell Medical Center, New York, NY
June-Aug 2014

Advisors: Dr. Fun-Sun Frank Yao, M.D. and Michele L. Steinkamp, RNS

- Analyzed coagulation efficiency of patients with various illnesses utilizing a ROTEM, and calculation and analysis were performed in excel.
- Obtained patient consent for clinical trial enrollment.
- Obtained and analyzed clinical research data and organized and maintained patient medical history.
- Investigated the effects of dexamethasone administered pre-operatively to patients undergoing Caesarian-sections by obtaining follow up PONV (Post-operative nausea vomiting) analysis

Anesthesiology Department Intern, Weill Cornell Medical Center, New York, NY
June-Aug 2014

Advisors: Dr. Fun-Sun Frank Yao, M.D. and Michele L. Steinkamp, RNS

- Learned the basics of anesthesia, such as the function of anesthesia machine and its ability to measure electrocardiogram (EKG), Heart rate, blood pressure and sustain proper oxygen saturation to utilize Desflurane to control the levels of propofol. Learned about many anesthetics and their functions, such as propofol, Succinylcholine, Fentanyl, Phenylephrine, etc.
- Understood anesthesiology pre-operative evaluations, such as obtaining patient medical history to determine if factors like sleep apnea, acid reflux, and past surgical history would impede tracheal intubation.
- Witnessed numerous specialized and complex procedures such as general surgery, interventional radiology, endoscopy, colonoscopy, acute pain management, obstetric anesthesia, kidney stone removal, cardiac surgery, caesarean section, and electroconvulsive therapy (ECT).

SELECTED PRESENTATIONS

- **Joshua Hsu.** Drug Discovery: Investigation of Drug Metabolism of compounds that target mutant p53. Presented at the North Jersey American Chemistry Society Section's 67th Annual Undergraduate Research Conference, Teaneck, NJ, 2015.
- **Joshua Hsu.** 8-Quinolyl-N-arylcarbamates as re-activators of mutant p53 and potential anti-tumor agents, MEDI 287, *Am Chem Soc.* Presented at the American Chemistry Society National Meeting, Philadelphia, PA, 2012.
- **Joshua Hsu.** Nuclear Keratin 17 and its role in the DNA Damage Response. Presented at the Johns Hopkins Bloomberg School of Public Health Department of Biochemistry & Molecular Biology Research Retreat, Gettysburg, PA, 2017.

PUBLICATIONS

THESIS

- **Master of Health Science (M.H.S.) Thesis**: Self-Assembled Aptamer Complexes as a Novel Therapeutic for targeted Alisertib delivery against Aurora A in Breast Cancer.
- **Master of Science (Sc.M.) Thesis**: Nuclear Keratin 17 and its role in the DNA damage Response.

ABSTRACTS

- 8-Quinolyl-N-arylcarbamates as re-activators of mutant p53 and potential anti-tumor agents, MEDI 287, *Am Chem Soc.* National Meeting, Philadelphia, PA, 2012.
- Ryan P. Hobbs, **Joshua Hsu**, Justin T. Jacob, Pierre A. Coulombe. "A role for nuclear-localized keratin 17 in the response of skin tumor keratinocytes to DNA damaging agents." Society for Investigative Dermatology. Portland, OR, 2017.
- **Joshua Hsu**, Ryan P. Hobbs, Pierre A. Coulombe. "Nuclear Keratin 17 and its role in the DNA Damage Response." Biochemistry & Molecular Biology Retreat. Gettysburg, PA, 2017.

WORK EXPERIENCE

Assistant, Residential School on Medicinal Chemistry and Biology, Madison, NJ **June 2015-July 2015**

- Assisted fellow residents throughout the residential school by organizing various events for smooth lectures.
- Attended several lectures on medicinal chemistry, subjected included: fragment-based drug design, clinical development, pharmacokinetics, enzymology, G-coupled protein receptors, Chemoinformatics, and case histories on drug development for many marketed drugs, such as eltrombopag and adcetris.

Subject Tutor, Drew University Center of Academic Excellence, Madison, NJ **Oct 2013-Mar 2014**

Cardinal Scholars, Madison, NJ **Feb 2014-Apr 2014**

- Tutored students in organic chemistry, biochemistry and molecular biology

Teaching Assistant, Drew University Chemistry Department, Madison, NJ **Sep 2013-Dec 2013**

- Assisted organic chemistry students in the laboratory, and ensured proper chemical waste disposal.

ALTRUISTIC EXPERIENCE

Case Manager, Charm City Clinic, Baltimore, MD **Feb 2016-Present**

- Provide guidance and assistance to patients in dire need of health insurance and a primary care physician.
- As a member of the health resource center (HRC), I also provide assistance to patients in need of various programs that aid with their alcoholism and drug abuses.

Administrative Volunteer, Shepard's Clinic, Baltimore, MD **Nov 2016-Present**

- Responsible for contacting patients for appointments and organizing patient data files.
- Assist in cleaning the clinic whenever possible, and encourage patients to sign up for various fun activities that the Joy and Wellness Center provides.

Head of Family Mentor, Thread, Baltimore, MD **Sep 2016-Present**

- Provide guidance to an assigned troubled student attending school in Baltimore city by directing reaching out to the students and their families, working with other Thread members on a plan to help students graduate high school and attend a four-year undergraduate institution.

Volunteer, Baltimore Rescue Mission Medical Clinic, Baltimore, MD **Sep 2015-Dec 2015**

- Assisted patients and local medical residents in diagnosing and treating homeless population in the Baltimore city area.
- Obtained past patient medical history for proper diagnosis.
- Utilized blood lancets to measure blood glucose levels.
- Assisted with patient physical check up by measuring blood pressure and auscultation.

Volunteer, Uni-med Family Health Care, Brooklyn, NY **June 2015-Aug 2015**

- Assisted patients in an underserved area by collecting blood work, utilizing phlebotomy and other venipuncture techniques.
- Obtained and analyzed cardiac electrical activity of patients using electrocardiogram (EKG).
- Assisted with patient physical check up by measuring blood pressure and auscultation.
- Analyzed CT scans to assist with patient cancer diagnosis.
- Performed vaccinations and vitamin B12 shots.

Volunteer, Drew University Civics Scholars Humanitarian Relief, Staten Island, NY **Oct 2012-Nov 2012**

- Assisted local residents affected by Hurricane Irene by organizing supplies for the homeless.
- Tore down and rebuilt damaged houses to recover personal property and ensure a safer environment.
- Delivered food and water.

Missionary Personnel, The Presbyterian Church in Taiwan, Taipei **June 2011-Jul 2011**

- Organized and reestablished church and tribal events for the local aboriginals of Hong-Ye.
- Cleaned elderly shelters and taught English to a class of children.

AWARDS AND HONORS

- Dean's Honor List 2012, 2013, 2014, 2015
- Drew University Dean's Award Scholarship
- Tri-Beta Honor Society (Upsilon Delta Chapter)
- Alpha Phi Omega (Pi Upsilon Chapter)

LABORATORY SKILLS

Biology Laboratory Techniques:

- Western blotting, PCR, qRT-PCR, qPCR, Nested PCR, SDS-PAGE, DNA/RNA isolation and purification, genome cloning, sequence analysis, chromosome spreads, and gel electrophoresis. Culture and aseptic techniques, gram staining, plasmid midiprep, serial dilution, LB agar medium preparation, bacterial transformation, bacterium inoculation, human microsome metabolism assay, *in vivo* tissue isolation, intraperitoneal (IP) injections, cryosectioning, hematopoietic stem cell isolation, splenocyte isolation, chemical inhibition assay, chemotaxis assay, MTT assay, stem cell culturing, tissue culture, migration assay, luciferase reporter assay, ELISA assay, histology analysis, tubule squashes, immunoprecipitation, immunofluorescence staining, lipophilic & optical transfection, comet assays, Mnase assay, chromosome spreads.

Chemistry Laboratory Techniques:

- Extraction, reflux, titration, filtration, vacuum filtration, distillation, TLC, flash column chromatography, affinity chromatography and recrystallization

Equipment Skills:

- Utilized various amount of research equipment such as a Rotary evaporator, fluorescence microscopy, UV-Vis spectrophotometer, spectrophotometer, LC/MS, GC/MS, NMR, IR, Chiral column Chromatography, affinity chromatography, streptavidin pulldown, flow cytometer, NanoDrop spectrophotometer and 2D-NMR (COSY), Confocal Microscope

Computer Programs Skills:

- ChemDraw, Java, Microsoft programs (World, PowerPoint, Excel, Excess), Adobe programs (Photoshop and Illustrator), SPSS, Masslynx, Scifinder, Zeiss imaging software, and Image J

Languages:

- Mandarin and Taiwanese

RELEVANT COURSES

Drew University:

Principles of Chemistry I
Principles of Chemistry II
Introduction to Psychology
Molecular and Cellular Biology
Organic Chemistry I
Organic Chemistry II
Microbiology
Calculus and Analytical Geometry I
Calculus and Analytical Geometry II
Introductory Statistics
Biochemistry I
Vertebrate Anatomy and Physiology I
Vertebrate Anatomy and Physiology II
Molecular Genetics
Chemical Biology
Biological Psychology
Bio-Medical Ethics
Virology
Molecular Biology of Cancer
Research in Biochemistry
Research in Chemistry
Immunology
Epidemiology & Biochemistry Capstone Seminar

Johns Hopkins Bloomberg School of Public Health:

Biochemistry II

Fundamentals of Reproductive Biology

Introduction to Molecular Biology

Molecular Toxicology

Evolution of Infectious Diseases

Fundamentals of Human Physiology

Principles of Cell Biology

Clinical Oncology

Immunology, Infection, and Disease

Molecular Biology of Pandemic Influenza

Pathogenesis of Bacterial Infections

Autoimmune diseases of the endocrine glands